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Choanoflagellates, choanocytes, and animal multicellularity

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Abstract. It is widely accepted that multicellular animals (metazoans) constitute a monophyletic unit, deriving from ancestral choanoflagellate-like protists that gave rise to simple choanocyte-bearing metazoans. However, a re-assessment of molecular and histological evidence on choanoflagellates, sponge choanocytes, and other metazoan cells reveals that the status of choanocytes as a fundamental cell type in metazoan evolution is unrealistic. Rather, choanocytes are specialized cells that develop from non-collared ciliated cells during sponge embryogenesis. Although choanocytes of adult sponges have no obvious homologue among metazoans, larval cells transdifferentiating into choanocytes at metamorphosis do have such homologues. The evidence reviewed here also indicates that sponge larvae are architecturally closer than adult sponges to the remaining metazoans. This may mean that the basic multicellular organismal architecture from which diploblasts evolved, that is, the putative planktonic archimetazoan, was more similar to a modern poriferan larva lacking choanocytes than to an adult sponge. Alternatively, it may mean that other metazoans evolved from a neotenous larva of ancient sponges. Indeed, the Porifera possess some features of intriguing evolutionary significance: (1) widespread occurrence of internal fertilization and a notable diversity of gastrulation modes, (2) dispersal through architecturally complex lecithotrophic larvae, in which an ephemeral archenteron (in dispherula larvae) and multiciliated and syncytial cells (in trichimella larvae) occur, (3) acquisition of direct development by some groups, and (4) replacement of choanocyte-based filter-feeding by carnivory in some sponges. Together, these features strongly suggest that the Porifera may have a longer and more complicated evolutionary history than traditionally assumed, and also that the simple anatomy of modern adult sponges may have resulted from a secondary simplification. This makes the idea of a neotenous evolution less likely than that of a larva-like choanocyte-lacking archimetazoan. From this perspective, the view that choanoflagellates may be simplified sponge-derived metazoans, rather than protists, emerges as a viable alternative hypothesis. This idea neither conflicts with the available evidence nor can be disproved by it, and must be specifically re-examined by further approaches combining morphological and molecular information. Interestingly, several microbial lineages lacking choanocyte-like morphology, such as Corallochytrea, Cristidiscoidea, Ministeriida, and Mesomycetozoea, have recently been placed at the boundary between fungi and animals, becoming a promising source of information in addition to the choanoflagellates in the search for the unicellular origin of animal multicellularity.

Additional key words: animal evolution, invertebrate larvae, metazoan ancestor, poriferan gastrulation, sponge development

I review here the evidence supporting the well-established hypothesis that animal multicellularity evolved from a unicellular choanoflagellate-like stage. I discuss some weaknesses of this hypothesis and suggest an alternative evolutionary pathway. In reviewing the anatomy and the embryology of the most basal metazoans, the Porifera, I found that some crucial aspects have long been misinterpreted and that inadequate knowledge has biased the analysis of sister-group relationships not only between the Porifera and other basal metazoans, but also between the Porifera and their putative protist ancestors, the choanoflagellates. Because of conflicts in the outcomes of the many cladistic analyses published to date on the origin and relationships of basal metazoans, the chances of a re-

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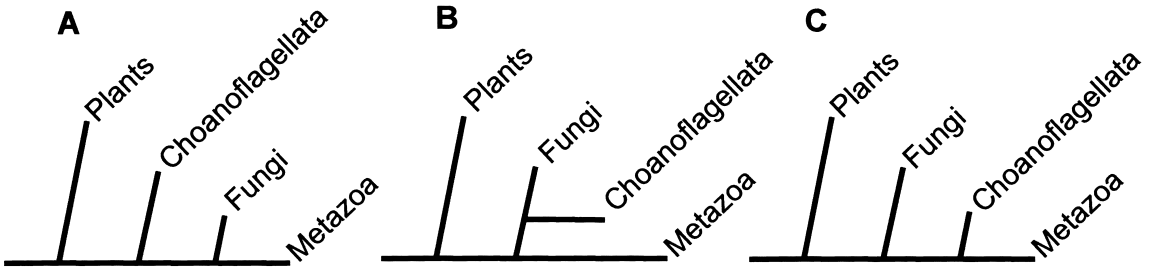

Fig. 1. Competing tree topologies showing the most plausible hypotheses for the phylogenetic relationships between Fungi, Choanoflagellata, and Metazoa.

liable cladistic test, either to falsify the established hypothesis or to validate any alternative one, seem minimal at present. Although cladistics presumably serves to standardize methods and facilitate testing and comparing different studies, substantial variety in methods of character selection, character coding, scoring and weighting, ground pattern reconstruction, and taxon selection can hinder comparison of different studies and can yield spurious phylogenetic trees and lack of consensus (Jenner & Schram 1999; Philippe 2000). Moreover, morphological and molecular phylogenetic trees are still often telling different stories. Therefore, before we can expect a cladistic test to clarify reliably the origin of metazoans, we need to re-assess the morphological evidence supporting any analyses. This is the main objective of my review, in the hope that its conclusions may help to redirect the perspective of future molecular and morphological cladistic approaches to the origin of metazoans.

The emergence of multicellular animals (Metazoa) was a crucial event in the history of life. Before the advent of molecular tools, zoologists concerned with the transition between the first metazoans and their putative unicellular ancestors were limited to searching through extant protists and lower metazoans for comparable structural traits by which to test their hypotheses (e.g., reviews by Salvini-Plawen 1978; Barnes 1983, 1985; Boero et al. 1998; Nielsen 2001). One of the most enduring connections between protists and metazoans was established over 130 years ago, when the remarkable similarity in cell architecture between choanoflagellate protists and choanocytes of Porifera (sponges) was realized (Clark 1866). This led to the hypothesis that the most primitive metazoans, the Porifera, evolved from choanoflagellate-like protist ancestors (Clark 1868; Haeckel 1874).

Initially, this cytological similarity was also used to support the opposite hypothesis, that sponges are colonial choanoflagellates (Clark 1868; Kent 1878). However, on the basis of spermatogenesis, Schulze established metazoan status for sponges in 1885. He also regarded the similarity between choanocytes and choanoflagellates to be mere analogy, but homology between both cell types has been confirmed by a series of comparative studies on structure and function over the last 40 years (Salvini-Plawen 1978; Afzelius 1961; Nielsen 1987). Consequently, the idea that Porifera arose from an ancestral choanoflagellate-like protist has progressively gained acceptance over competing hypotheses. Concurrently, the monophyly of metazoans has slowly emerged with the accumulation of consistent results from ultrastructural, biochemical, and genetic studies (Salvini-Plawen 1978; Barnes 1985; Ax 1989; Wainright et al. 1993; Kumar & Rzhetsky 1996; Ragan et al. 1996; Müller 1998; Lang et al. 2002). If we accept the monophyly of metazoans, the evolutionary role postulated for the choanoflagellate-like protists automatically expands from putative ancestors of just the sponges to ancestors of all metazoans. During the past 10 years, most efforts to test the reliability of this shift in the role of choanoflagellates were based on small subunit (SSU) rDNA sequences, and the analysis of the relationships within the Opisthokonta was the first serious challenge.

Opisthokonta was initially proposed (*sensu* Cavalier-Smith 1988) to be a cohesive evolutionary supergroup, which contained animals (Metazoa) and their putative protist relatives (the choanoflagellates) and true fungi and their putative protist relatives (the chytrids), because they all share flattened mitochondrial cristae and a single posterior cilium/flagellum[1] on reproductive cells. Phylogenetic analyses based on SSU rDNA sequences have corroborated that these groups are somehow related, but produced inconsistent patterns of relationship within the supergroup. While

[1] I use the term ''cilia'' to refer to eukaryotic organelles whose structure is characterized by an essentially identical arrangement of microtubules. Following Nielsen (2001), this definition covers a spectrum from the undulating cilium of many protists and sperm cells to the planar cilium of vertebrate multiciliated cells. I reserve the term flagellum for simpler structures found in bacteria, which lack microtubules.

some studies placed choanoflagellates basal to the animal-fungal split (Fig. 1A; e.g., Cavalier-Smith 1987; Van de Peer & De Watcher 1997), most others depicted alternative relationships between choanoflagellates, other protists, fungi, and metazoans (Fig. 1B,C), also conflicting as to the relative derivation of the lower metazoan groups (e.g., Wainright et al. 1993; Kumar & Rzhetsky 1996; Cavalier-Smith et al. 1994; Smothers et al. 1994; Ragan et al. 1996; Müller 1998; Van de Peer et al. 2000; Medina et al. 2001). Uncertainty about relationships within Opisthokonta has been aggravated recently. Apart from choanoflagellates and chytrids, several other microbial taxa (i.e., nucleariid amoebas, mesomycetozoeans, *Ministeria,* and *Corallochytrium*) traditionally held to be fungi, algae, or protozoans, have been shown to have mitochondria with flat cristae, and are also placed at the boundary between fungi and animals by molecular phylogeny (Mendoza et al. 2002; Cavalier-Smith & Chao 2003). However, the exact pattern of relationships between these groups remains unresolved.

The problems of SSU rDNA approaches to the complete resolution of relationships within the Opisthokonta are attributed mostly to variable rates of evolution and base composition effects (e.g., Kumar & Rzhetsky 1996; Van de Peer & De Watcher 1997; Medina et al. 2001; Cavalier-Smith & Chao 2003). These problems have prompted a complementary approach: the sequencing of nuclear genes coding for very conservative eukaryotic proteins, such as heat-shock proteins (Hsp 70), elongation factors (EF-2), and alpha-tubulin (Snell et al. 2001; King & Carroll 2001). These sequences support the hypothesis that choanoflagellates are more closely related than fungi to metazoans (Fig. 1C). Moreover, the gap between metazoans and choanoflagellates narrowed considerably after the discovery that a species of unicellular choanoflagellates possesses receptor tyrosine kinase, a highly conservative protein system involved in cell-to-cell communication and adhesion, which had not been found before outside Metazoa (King & Carroll 2001). However, it remains perplexing that, whereas the putative transition between choanoflagellates and sponges—theoretically occurring during the Ediacaran—is still reflected in a striking resemblance in cell architecture, there is no such evidence for the subsequent morphological transition between sponges and the remaining metazoans. Nor is there any evidence for a putative morphological transition between the choanoflagellates and other protists. I contend that the reason for such discontinuities in organismal architecture is that, contrary to current opinion, those transitions did not occur, at least not in the way traditionally postulated.

The Obscure Transition Between Sponges and Higher Diploblasts

The body of adult sponges, which are capable of cell dissociation and re-aggregation, preserves traces of a colonial-like organization and possesses a variety of distinctive cytological features not found in other metazoans, the presence of choanocytes included (Simpson 1984; Barnes & Harrison 1991; Nielsen 2001). Adult sponges also lack traits that occur in other metazoans, including nerve cells, sensory organs, muscle cells, epithelia with basement membranes (except for basement membranes known in 1 sponge species), belt desmosomes, a true endodermal cavity (archenteron), and arguably, a true endodermal layer (e.g., Simpson 1984; Barnes & Harrison 1991; Nielsen 2001). This architectural gap between sponges and the remaining metazoans is puzzling because it challenges most recent molecular phylogenies, which consistently find Metazoa to be monophyletic (e.g., Kobayashi et al. 1996; Borchiellini et al. 1998; Müller & Müller 1999). Indeed, sponges possess DNA sequences coding for a variety of molecules also found in the remaining metazoans, such as integrin, fibronectin, galectin, tyrosine kinase receptor, serotonin, crystallin, metabotropic glutamate receptor, and immunoglobulin-like molecules (reviewed in Müller 1998).

Despite a number of molecular approaches, usually based on 18S rRNA sequences, the relationships between Porifera and other diploblasts are unresolved: the resulting phylogenies often conflict with each other and with traditional views on the early evolution of Metazoa. Until the 1970s, most authors agreed on the monophyly of Porifera (but see Gray 1867), based on the morphological evidence available at the time. However, after the discovery that hexactinellid sponges have syncytial structures, some authors claimed that they stand clearly apart from the remaining sponges (Calcarea + Demospongiae), which are cellular (Reiswig 1979; Reiswig & Mackie 1983; Bergquist 1985). The names Symplasma and Cellularia (Reiswig & Mackie 1983) or Hexactinellida and Pynacophora (Mehl & Reitner 1996) were proposed to denote respectively such putative phyla.

The advent of molecular tools aggravated the uncertainty rather than helping to resolve relationships within Porifera (reviews: Cavalier-Smith et al. 1996; Lipscomb et al. 1998; Kim et al. 1999; Adoutte et al. 2000; Borchiellini et al. 2000). Some molecular studies have indicated that sponges are not monophyletic, because calcareous sponges (Calcarea) are more closely related to ctenophores than to siliceous sponges (Demospongiae + Hexactinellida), resuscitating an early proposal by Gray (1867) to distinguish between

Calcarea and siliceous sponges. In contrast, other studies have depicted a monophyletic Porifera as closer to Cnidaria or Placozoa than to Ctenophora. To complicate matters, in a phylogenetic analysis involving unicellular and metazoan organisms and based on sequences of cDNA encoding a protein kinase C (cPKC), Hexactinellida (represented by *Rhabdocalyptus dawsoni*) was the sister group to the remaining metazoans, including a clade of Demospongiae (represented by *Geodia cydonium* and *Suberites domuncula*) and a clade of Calcarea (represented by *Sycon raphanus*) + the higher invertebrates in the analysis. That is, *Drosophila melanogaster* and *Lytechinus pictus* appeared most closely related to calcareous sponges (Kruse et al. 1998). In contrast, a recent study by Cavalier-Smith & Chao (2003) presents distance trees, in which hexactinellids are sister to demosponges, as well as maximum likelihood trees, in which hexactinellids fall within demosponges as sister to a clade containing non-spiculate demosponges. Because hexactinellids are usually characterized in the trees by a long branch, the authors suggest that their usual exclusion from demosponges may be a long-branch artifact, concluding that Demospongiae is ancestral to Hexactinellida and thus paraphyletic (Cavalier-Smith & Chao 2003). However, it remains unclear how the distinctive syncytial organization and heavily silicified skeletons of hexactinellids evolved from an ancestor shared with non-spiculose, cellular demosponges.

These various competing and irreconcilable outcomes of the different phylogenetic analyses have created a climate of uncertainty rather than helping to resolve relationships within Porifera. I concur with Reiswig (2002) that molecular analyses have not yet generated consistent strongly supported relationships among poriferan classes (e.g., Collins 1998; Kruse et al. 1998; Adams et al. 1999; Borchiellini et al. 2001; Medina et al. 2001) and are unlikely to influence the systematic organization at high taxonomic levels for some time. Therefore, I advise approaching this review by adopting the attitude recently expressed by Hooper et al. (2002): ''We prefer, at this juncture of uncertainty, to avoid the issue of potential paraphyly within the Porifera altogether, until the matter has been more satisfactorily resolved.''

Molecular phylogenies, however, agree about the monophyly of metazoans and the basal placement of Porifera, which consistently branch off before the remaining clades (Wainright et al. 1993; Cavalier-Smith et al. 1996; Kumar & Rzhetshy 1996; Kim et al. 1999). Therefore, if Metazoa is a monophyletic unit, we should, sooner or later, be able to discern how sponges diverged morphologically from the remaining groups. A failure to grasp the evolutionary significance of some embryological processes and larval structures may be the cause of current problems in understanding how Porifera relate to other diploblastic metazoans.

Reinterpreting the Significance of Gastrulation in Porifera

A fundamental obstacle to establishing a clear correspondence between the histology of sponges and other diploblasts is the uncertainty surrounding the occurrence of gastrulation in Porifera. Because the external ciliated cells of some larvae internalize during development and differentiate into adult choanocytes (Delage 1892; Duboscq & Tuzet 1937; Lévi 1956; Lévi & Porte 1962; Borojevic 1966; Boury-Esnault 1976; Amano & Hori 1998), sponge larvae have usually been interpreted as a blastula stages, and gastrulation as coinciding with larval metamorphosis. Such an idea, disseminated in classical reviews and textbooks, is deeply rooted among zoologists (Brien 1967, 1973; Fell 1974; Simpson 1984; Barnes & Harrison 1991; Ruppert & Barnes 1994). More importantly, it presents sponges as anomalous metazoans with ''inversion of layers,'' in which the endodermal cells, unlike those in the remaining metazoans, derive from external embryonic cells. Therefore, while all major gastrulation modes in lower metazoans have long been well described, there are still reasonable doubts as to how and when gastrulation occurs in Porifera (reviews: Efremova 1997; Leys & Degnan 2002). I contend that poriferan gastrulation is not essentially different from that in other invertebrates (see also Leys & Degnan 2002). Development of 7 larval types out of 8 described in Porifera so far (Maldonado & Bergquist 2002) appears to involve cellular reorganizations that can be equated to typical gastrulation modes known from other invertebrates (Fig. 2).

The larva of most sponges in the class Hexactinellida remains unknown, but recent findings on a cave species have revealed that its trichimella larva is a post-gastrulation stage (Boury-Esnault et al. 1999). Cleavage leads to a coeloblastula, which at the 32-cell stage consists of a single layer of equal-sized blastomeres. Then, gastrulation proceeds by delamination, with the blastomeres dividing tangentially. Each division yields an external micromere and an internal macromere, producing a 2-layered, hollow embryo (Fig. 2). After gastrulation, histogenesis produces a solid, distinctive larva showing, among other features, multiciliated cells, several types of multinucleated cells, and non-functional choanochambers.

In some species of Halisarcida, demosponges of uncertain affinity that lack both mineral and spongin skeletons and may represent either a highly derived or

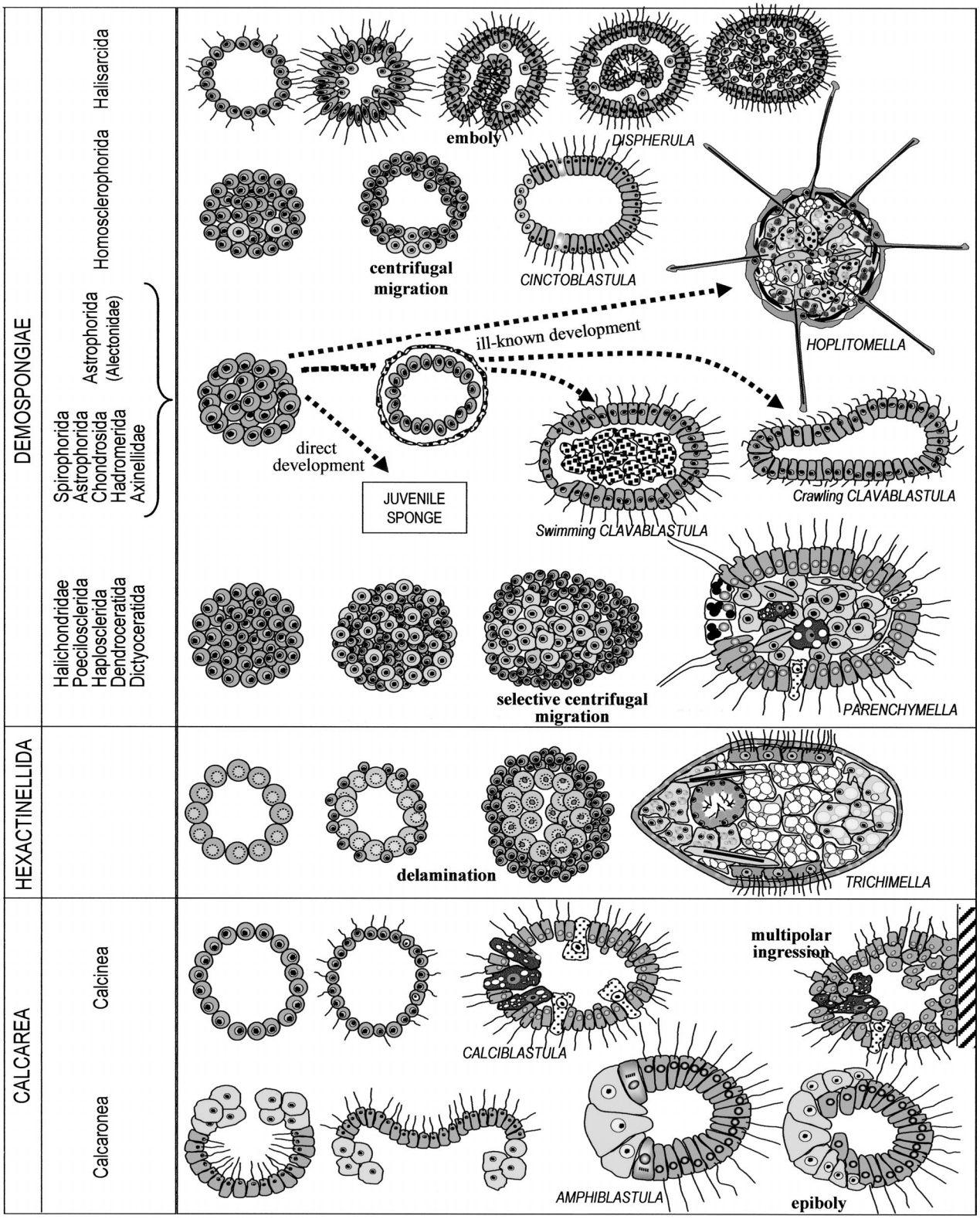

Fig. 2. Summary of cell rearrangements during embryogenesis in the Porifera. Cell re-arrangements that can be equated to gastrulation are labeled in bold; larval stages are labeled in uppercase. Arrows indicate developmental processes not detailed in the figure.

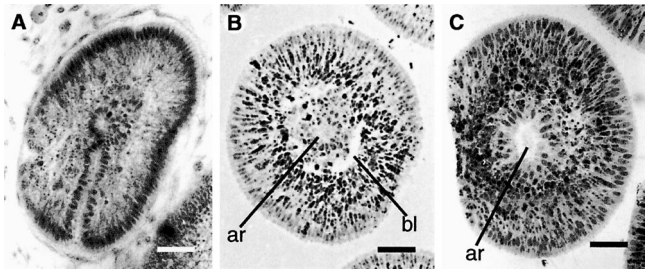

Fig. 3. Stages of development in *Halisarca dujardini*. **A.** Longitudinal section of embryo showing the posterior-lateral invagination typical of an embolic gastrulation. Scale bar, 25 μm. **B.** Transverse section of early dispherula stage, showing the ephemeral archenteron (ar) and a narrow blastocoel (bl). Scale bar, 20 μm. **C.** Transverse section of a late larva, in which the blastocoel has already been obliterated but the archenteron (ar) remains unfilled. Scale bar, 20 μm. (Micrographs by A. Ereskovsky)

a primitive group, gastrulation also occurs before larval differentiation. First, a few cells migrate from the epithelium of the coeloblastula into the blastocoel. These cells do not fill the blastocoel, but remain peripheral and close to the overlying epithelium without dividing or proliferating. The posterior-lateral ciliated blastoderm then invaginates (Figs. 2, 3). The invaginated blastomeres reorganize into a monolayered, internally ciliated tube, which may be straight or C-shaped; it remains suspended in a fluid-filled blastocoel (Figs. 2, 3). This stage is known as a dispherula larva. The lumen of the tube and the blastocoel in which the tube is contained are transitory cavities. They are closed, either during free-swimming larval life or at settlement, by proliferation of the few cells internalized before the emboly, as well as by late cell migrations (Figs. 2, 3). Several authors describing this development (Lévi 1956; Chen 1976; Harrison & De Vos 1991; Ereskovsky & Gonobobleva 2000) did not recognize gastrulation by emboly, one of the most fundamental gastrulation modes leading to the formation of the archenteron in other lower metazoans (Buss 1987). Yet, if the outer cavity corresponds to a blastocoel, as traditionally assumed, the internal cavity, by its aspect, origin, and position, can be nothing other than the homologous ephemeral rudiment of a truly endodermal cavity (archenteron), the first identified in Porifera (Maldonado & Bergquist 2002). Indeed, it strongly resembles the internally ciliated gastrovascular cavity described in the lecithotrophic planula of the scyphozoan *Aurelia aurita* (Martin & Koss 2002).

The larva of many species in the class Demospongiae, the parenchymella, has also been reinterpreted recently as a post-gastrulation stage in which no inversion of layers occurs. The late stereoblastula differentiates intermingled micromeres and macromeres, followed by a selective ''centrifugal migration'' (Borojevic & Lévi 1965) of micromeres towards the surface (Fig. 2). This long-known process has now been interpreted as gastrulation by ''mixed delamination'' (Leys & Degnan 2002). Then, cell differentiation begins to produce a solid parenchymella made of macromere-derived unciliated cells and micromere-derived ciliated cells, up to a total of 11 cell types (unpubl. obs.), occasionally including choanocytes organized into non-functional chambers. The free-swimming larva attaches to a substrate, and the ciliated micromeres of the larval wall migrate inwards during metamorphosis. They become both choanocytes and amoeboid cells of the adult. Therefore, these internal adult cells derive from internal embryonic cells that become transiently external during the larval stage. The idea of a larva made of fully differentiated cells is also consistent with observations that cells dissociated from a parenchymella larva re-aggregate directly to form a juvenile sponge rather than a larva-like organism (Borojevic & Lévi 1965).

Pertinent here are the results of a study by Misevic et al. (1990) on the metamorphosis of a parenchymella larva, in which the authors concluded that ciliated larval cells do not become choanocytes in the adults, but are engulfed and digested by archeocytes immediately after metamorphosis. After labeling the epithelial larval cells with a radioactive marker (1 mCi $Na^{125}I$ for 5 min), Misevic et al. (1990) observed that the marker was initially recovered in archeocytes surrounding ciliated cells internalized during metamorphosis. In a subsequent stage, the marker accumulated entirely in the archeocytes. The authors interpreted these results as evidence that the epithelial cells are normally phagocytized by archeocytes. However, archeocytes are the main constituents of the sponge immune system, and one of their functions is to phagocytize and neutralize foreign invasive bodies or unusual compounds, such as $Na^{125}I$. The authors assumed that a selective transfer of the marker from the ciliated cells to the archeocytes was unlikely. However, selective cell-to-cell transfer of vacuoles is not only common within the sponge body, but also the main mechanism by which ingested food is exchanged between cells. Therefore, the conclusion of Misevic et al. (1990) that the ciliated larval cells are destroyed after metamorphosis of the parenchymella is controversial at least. Moreover, studies that traced the post-metamorphic fate of natural markers in larval ciliated cells, such as characteristic electron-dense ellipsoid granules and phagosomes with remains of resorbed cilia (Boury-Esnault 1976; Amano & Hori 1998), recovered these markers in the choanocytes of early post-larval stages,

evidence that ciliated larval cells transformed into choanocytes.

Contrary to some recent interpretations (Ereskovsky & Boury-Esnault 2002), gastrulation may also precede larval differentiation in homosclerophorid sponges. The late stereoblastula, which consists mostly of micromeres with a few macromeres, becomes hollow through a combined process of histolysis and outward migration of micromeres (Meewis 1938; Tuzet & Paris 1964; Ereskovsky & Boury-Esnault 2002). This hollowing process, recently referred to as ''multipolar egression'' (Boury-Esnault et al. 2003), is a cell rearrangement similar to the gastrulation described for the stereoblastula of some cnidarians (Tardent 1978; Nielsen 2001), and I interpret this process as a true gastrulation (Fig. 2). Subsequent cell proliferation and differentiation lead to a ''gastrular'' larva consisting of a mono- or pseudostratified cell layer that surrounds an internal cavity filled with an intercellular matrix rich in collagen fibrils and symbiotic bacteria. These fibrils form a subepithelial felt, which has been interpreted as a true basement membrane (Boury-Esnault et al. 2003).

Depending on the species, the larva in Homosclerophorida is entirely ciliated or has a small posterior unciliated region derived from the few internal macromeres in the stereoblastula (Tuzet & Paris 1964). The ciliated cells of the equatorial region possess a paracrystalline intranuclear inclusion. These cells, which are seen as a refringent equatorial band through the light microscope, inspired the name of cinctoblastula larva (though ''cinctogastrula'' may be a better name). Interspersed among the remaining epithelial cells are also flask-shaped cells filled with vesicles (Lévi & Porte 1962; Boury-Esnault et al. 2003). During metamorphosis, the posterior cells proliferate over the anterior cells, which internalize to become choanocytes and other internal cells of the adult; the refringent cells form the excurrent canal system and posterior cells develop into the external pinacocytes of the adult (Meewis 1938). Therefore, all larval cells appear to have a predetermined fate, as is typical of a post-gastrulation stage.

Developmental information on alectonid demosponges is still insufficient to understand how gastrulation proceeds (Fig. 2). However, there is little doubt that their long-lived hoplitomella larva, erroneously regarded as an asexual propagule until recently (Vacelet 1999), is a post-gastrulation stage. Otherwise, it would be hard to explain the great variety of highly specialized cells that this larva contains: unciliated pinacocyte-like cells at the surface, collencytes (secreting collagen), at least 2 types of sclerocytes (secreting spicules of several types and sizes), choanocytes arranged in non-functional chambers, totipotent archeocytes, and diverse cell types with inclusions (Garrone 1974). The larva also has a more complex spicule skeleton than the adult. The larval spicules allowed the transfer of alectonid sponges from Hadromerida to Astrophorida (Maldonado & Bergquist 2002), an order in which no larval stage had been described.

Only in calcareous sponges does the larva appear to be a blastula stage, with gastrulation following larval differentiation (Fig. 2). In calcaroneous calcareous sponges, cleavage leads to an internally ciliated stomoblastula that everts to become an externally ciliated coeloblastula, which represents the definitive larva, an amphiblastula. The larval epithelium consists of monociliated micromeres in its anterior half, unciliated macromeres in its posterior half, and 4 cross cells equally spaced around the equator of the larval body. At the end of free-swimming period, the macromeres progressively overgrow the ciliated micromeres, internalizing the locomotory ciliated field. This process is identical to gastrulation by epiboly, after which the micromeres differentiate into choanocytes and internal amoeboid cells, while the unciliated macromeres, which remain external, differentiate into adult pinacocytes (Minchin 1896; Duboscq & Tuzet 1937; Amano & Hori 1993).

The larva of calcineid calcareous sponges, the calciblastula, appears to be a coeloblastula. Depending on the species, the monostratified blastoderm consists either entirely of externally monociliated cells of equal size or, in addition, a few (2–10) large, unciliated cells located at the posterior larval pole. Towards the end of larval dispersal, cells of the blastoderm lose the cilium and move into the blastocoel. Migration involves many of the ciliated cells (Minchin 1900; Tuzet 1948; Borojevic 1969; Amano & Hori 2001), but it remains unclear whether the few unciliated posterior cells participate in this process. This cell migration appears to correspond to typical gastrulation by multipolar ingression (Fig. 2). Subsequently, internalized cells differentiate into choanocytes and various amoeboid cell types of the adult mesohyl, whereas those remaining in the larval blastoderm form the external pinacocytes of the adult. The fate of the unciliated macromeres remains unclear (Amano & Hori 2001).

The embryology of several sponge orders (Astrophorida, Chondrosiida, Hadromerida, Axinellida), in which development usually takes places externally within a few hours, needs further investigation. The larva of these orders, provisionally referred to as a clavablastula (Maldonado & Bergquist 2002), is currently regarded as a coeloblastula. The information available on these larvae does not yet explain how the solid morula becomes a coeloblastula (Fig. 2). The lar-

val blastoderm is monolayered and entirely ciliated. In some groups (e.g., *Chondrosia*), the blastocoel may be secondarily filled by maternal cells, on which the developing embryo feeds (Lévi & Lévi 1976).

In some species of Spirophorida and Hadromerida, there is no larva and the embryo develops directly into a juvenile sponge (Fig. 2). From the information published to date, no cell movements equivalent to a gastrulation can be identified. However, it appears that all embryonic stages are unciliated and apparently none can be equated to a reduced larva. The best studied spirophorids are several species in the genus *Tetilla.* These sponges are gonochoric and release gametes into the sea. The eggs are provided with radiating bundles of collagen fibers, which may inhibit sinking. Upon external fertilization, the eggs produce a fertilization membrane that encloses the radiating collagen fibers in the perivitelline space (Watanabe & Masuda 1990). The fertilized egg, provided with a sticky surface, adheres to the substrate and undergoes cleavage, which is total and nearly equal, resulting in a solid blastula (Watanabe 1960). At this stage, some of the outer cells form a protrusion and contact the substrate. Soon other cells migrate to the contact area, and the protruding multicellular structure attaches the developing embryo to the substrate. Some cells on the attached side of the embryo begin to migrate inward, forming a depressed area. It remains unclear whether such cell movement is a peculiar form of gastrulation or part of ''organogenesis.'' Formation of the depressed area appears to mark the start of differentiation, as macroscopic fiber-like structures are seen in the depressed area producing the primordium of the root system by which the adult sponge will attach to the substrate.

At this stage, sclerocytes also differentiate from cells in the core of the developing embryo and initiate spicule secretion. Some of the cells that migrated inside the embryo appear to give rise to the choanocyte chambers. Meanwhile, incurrent canals appear, the number and size of spicules in the inner portion of the embryo increase, and the radiating skeleton that characterizes the adult sponges is formed. Once incurrent canals are present, the external cells of the embryo flatten and differentiate into pinacocytes, and the incurrent pores also appear. The excurrent canal system and a single osculum are a final step in the production of a functional juvenile sponge. Likewise, direct development has been reported in the deep-sea hadromerid demosponge *Stylocordyla borealis* (Sarà et al. 2002). The eggs are brooded within the maternal body until they become functional juvenile sponges provided with choanochambers and a complete set of spicules. Because no sperm have been seen, it has been hypothesized that these eggs may develop parthenogenetically (Bergquist 1972).

In summary, despite the scarcity of information for some taxonomic groups, gastrulation in Porifera appears to follow recognizable models (Fig. 2), taking place immediately after cleavage and before larval differentiation in most cases, but not in all calcareous sponges. This means that most sponge larvae are formed of differentiated cells rather than totipotent blastomeres. There is now evidence for the 2 subclasses of Calcarea and several groups of Demospongiae that larval ciliated cells, which are not only fully differentiated but also show a structure homologous to that of other metazoan ciliated cells, ''transdifferentiate'' during larval metamorphosis into the distinctive choanocytes of adult sponges. Because the number of choanocytes in a sponge is clearly higher than the number of ciliated cells in any larva, new choanocytes must arise by conventional mitosis in juveniles.

Reinterpreting the Significance of the Larval Histology

Further evidence that most sponge larvae are post-gastrulation stages consisting of differentiated cells is that larvae usually have more sophisticated architecture than adults. A clear example is the structure of the epithelia. The surface epithelium of adult sponges is known as the pinacoderm. Unlike the epithelia of other metazoans, the pinacoderm consists of weakly polarized cells that may routinely migrate into the inter-epithelial mesenchyme because there is no basement membrane (e.g., Simpson 1984; Barnes & Harrison 1991; Nielsen 2001). An exception is the dense accumulation of type IV collagen, the basic element of basement membrane, found beneath the pinacoderm of a homosclerophorid species (Boute et al. 1996). In contrast, larval epithelia usually contain several cell types with marked polarity (Fig. 4A), and possess basal collagen reinforcements more often than is generally reported. For instance, abundant subepithelial collagen has recently been reported in the hoplitomella larvae of alectonids (Vacelet 1999). The presence of fibrous mesenchyme beneath the epithelium of homosclerophorid sponges has also long been known from studies on their larval stage (Lévi & Porte 1962), and such a structure has recently been interpreted as a true basement membrane consisting of a subepithelial feltwork of collagen fibrils underlain with a loose network of collagen fibrils (Boury-Esnault et al. 2003). Likewise, the epithelium of the parenchymella larva of the poecilosclerid demosponge *Crambe crambe* has a distinctive, delicate, basal network of fibrils (Fig. 4B,C). We cannot discard the hypothesis that subepithelial net-

works or similar subtle collagen reinforcements are likely to occur in many other larvae, having gone unnoticed in previous TEM studies.

Information derived from the study of cell junctions, usually used in discussions concerning the evolutionary status of animal phyla (Green & Bergquist 1982; Nielsen 2001), is equivocal as to whether larvae or adults are more complex. So far, no type of cell junction involved in the process of rapid chemical or electrical intercellular communication has been reported in sponges, except for the mere suggestion that cytoplasmic bridges between putative photoreceptor cells of a parenchyemella larva may serve this purpose (Maldonado et al. 2003). Only simple electron-dense junctions, parallel junctions, and septate junctions have been described in the phylum. Even these types are usually difficult to visualize because, due to the transient nature of virtually all intercellular interactions in sponges, they are ephemeral and are formed only when required for specific functions (Green & Bergquist 1979). Nonetheless, parallel junctions that resemble the ''zonula adherens'' of the desmosome and may represent an early stage in the evolution of desmosomes have been described between the subepithelial multiciliated cells of the trichimella larva of hexactinellids (Boury-Esnault et al. 1999) and also between ciliated cells of the parenchymella larva of a demosponge (Rieger 1994a). True desmosomes have been reported in the cinctoblastula larva of homosclerophorid sponges (Boury-Esnault et al. 2003). In adults, parallel junctions have been described between the cells forming the ''epithelium'' of the brood chamber of some demosponges (Green & Bergquist 1979), and also between the contractile actin-bearing pinacocytes surrounding the oscules of some demosponges, in which desmosome-like structures (not true desmosomes) occur (Masuda et al. 1998). Occluding junctions of septate type occur between the collar bodies and the trabecular tissue of hexactinellids (Mackie & Singla 1983). In demosponges, they have been found between choanocytes (Green & Bergquist 1979; Alves de Matos et al. 2002) and spongiocytes (De Vos 1977). In calcareous sponges, septate juctions have been reported between sclerocytes (Ledger 1975). In contrast, septate junctions remain unknown in sponge larvae.

A comparison of the ultrastructure of ciliated cells again supports the higher complexity in larvae vs. adults. Ciliated cells of adult sponges consistently bear a single cilium. This is consistent with the observation that all diploblasts and most deuterostomes possess monociliated cells, multiciliated cells being acquired later in animal evolution (e.g., Barnes 1985; Nielsen 2001). Some sponge larvae, however, do not follow this rule. Occasional bi-ciliated cells occur in larval epithelia (Fig. 4D). Although they are postulated to result from defective cell divisions (Lévi 1964), their origin and significance remains uncertain (Maldonado et al. 2003). Furthermore, recent observations on the trichimella larva of hexactinellids have revealed a complex, unique epithelial organization (Boury-Esnault & Vacelet 1994; Boury-Esnault et al. 1999). The locomotory ciliation of this larva is provided by subepithelial multiciliated cells, the cilia of which reach the exterior only after piercing an unciliated membrane-like syncytial ''epithelium'' (Fig. 5A).

Adults and larvae also differ in ciliary ultrastructure. Ciliated cells of most metazoans possess a system of rootlets that originates from the ciliary basal body and extends into the cytoplasm. Ciliary rootlets usually show a characteristic cross-striation. In contrast, rootlet systems are lacking in ciliated cells of adult sponges (i.e., choanocytes, collar bodies, and ciliated pinacocytes of some sponges), but they occur in monociliated cells of the larval epithelia (Figs. 4E, 5). The rootlets of most larvae in the class Demospongiae lack the cross-striation characteristic of Eumetazoa (Figs. 4E, 5C), except for larvae in the order Homosclerophorida (Boury-Esnault et al. 2003) and the larva of a poecilosclerid sponge (Lévi 1964). Striated rootlets (Fig. 4B) consistently occur in the cilia of calcareous sponge larvae (Gallisian 1983; Woollacott & Pinto 1995; Amano & Hori 2001). Rootlets appear to be absent in multiciliated cells of the only hexactinellid larva studied so far (Fig. 5A) (Boury-Esnault & Vacelet 1994; Boury-Esnault et al. 1999).

Yet there are more differences between ciliated cells of larvae and adults. The ciliary basal body in most metazoans is connected to 9 alar sheets and their respective anchor points, a system that fixes the base of the cilium to the surrounding cell membrane. These structures (Figs. 4F–H, 5A–C) also occur in both monociliated (Woollacott & Pinto 1995; Boury-Esnault et al. 2003; Maldonado et al. 2003) and multiciliated (Boury-Esnault et al. 1999) cells of poriferan larval epithelia, but they are absent in ciliated cells of adult sponges.

The larval cilia may also be involved in sensory processes. Ciliated cells of the posterior pole of many parenchymella larvae bear a long cilium and peculiar, distal protrusions filled with pigment inclusions and mitochondria (Fig. 4I). These long posterior cilia detect light and modify the swimming trajectory of the larva in response to changes in light intensity (Maldonado & Young 1996; Leys & Degnan 2001). The structure, arrangement, and cooperative behavior of the putative photoreceptor cells make the posterior pole of the larva function as a photoreceptor organ-like structure (Maldonado et al. 2003). In contrast,

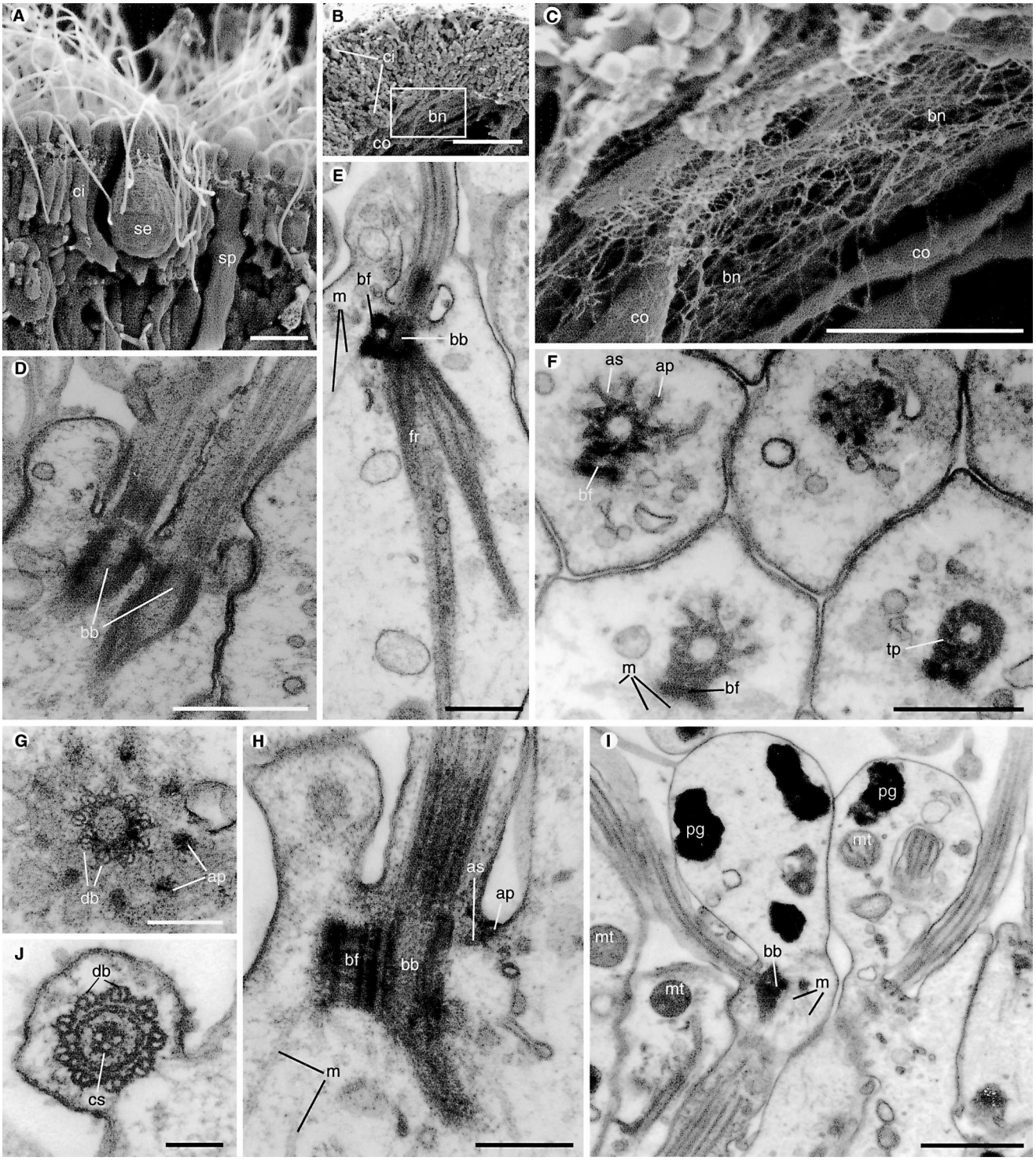

Fig. 4. Cytological details of larval stages of sponges. **A.** Larval epithelium of poecilosclerid demosponge *Mycale* sp., showing ciliated (ci), secretory (se), and spherulous (sp) cells. SEM. Scale bar, 5 μm. **B.** Transverse section of larval epithelium (ci) of poecilosclerid demosponge *Crambe crambe.* The epithelium is lined by a basal collagen network (bn) and tangential collagen-secreting cells called collencytes (co). SEM. Scale bar, 20 μm. **C.** Detail of the basal collagen network and collencytes. Scale bar, 10 μm. **D.** Longitudinal section of a biciliated cell in larval epithelium of haplosclerid demosponge *Sigmadocia caerulea,* showing 2 basal bodies (bb). Scale bar, 500 nm. **E.** Longitudinal section of monociliated cell of larval epithelium of *S. caerulea,* showing basal body (bb), a branched, non-striated rootlet (fr), and a basal foot (bf) from which microtubules (m) project. Scale bar, 1 μm. **F.** Transverse section of epithelial cells in larva of *S. caerulea,* showing the basal bodies of microtubule triplets (tp), and surrounded by 9 alar sheets (as) and anchor points (ap). Basal

adult sponges do not have any known sensory organs. Adult sponges perceive environmental stimuli or manifest elemental behavioral responses (e.g., Leys & Mackie 1997; Maldonado & Uriz 1999). However, behavior patterns result from stimulation and response of individuals cells, rather than from occurrence of specialized sensory cells or organ-like structures.

In addition, the fine structure of some larval cilia suggests a potential connection between sponge larvae and some cnidarians. As in most invertebrates, the axoneme of all known cilia in adult sponges has a typical "$(9 \times 2) + 2$" structure. In contrast, the basal portions of cilia in the parenchymella larva may contain 3 central microtubule singlets (Fig. 4J). This "$(9 \times 2) + 3$" organization is extremely uncommon in animals, known only from the non-motile photoreceptive cilia of another diploblast, the cnidarian polyp of *Stylocoronella riedli* (Blumer et al. 1995). The situation is even atypical for Cnidaria, as ocelli are typically restricted to the medusa stage. It is possible that this third central singlet occurs in other sponge larvae and cnidarians, but has escaped previous TEM observations.

In summary, sponge larvae appear closer than adult sponges to the "eumetazoan" level of organization, a conclusion consistent with previous suggestions that homologues of metazoan structures are more likely to be found in larval than in adult stages (e.g., Haeckel 1874; Salvini-Plawen 1978; Nielsen 1994; Rieger 1994b; Weyrer et al. 1999). Such an architectural relationship may mean that other metazoans evolved by neoteny from an early developmental stage of ancient sponges. Alternatively, it may mean that the basic multicellular organismal architecture from which diploblasts evolved, that is the putative planktonic archimetazoan, was more similar to a modern poriferan larva lacking choanocytes than to an adult sponge.

Several traits suggest that the Porifera may have a longer evolutionary history than traditionally assumed. Otherwise, it would be hard to explain the unexpected diversity of gastrulation modes and lecitothropic larval types, the occurrence of multiciliated and syncytial cells in the larva of hexactinellids, the presence of an ephemeral archenteron in the dispherula larva of halisarcid sponges, the acquisition of direct development in some groups, and the widespread occurrence of internal fertilization. Indeed, it is particularly surprising that all known sponge larvae are lecithotrophic, although planktotrophy is assumed to be the primitive condition for the larvae of most invertebrate phyla (Strathmann 1985, 1993; Pechenik 1999).

Similarly, the occurrence of direct development in some sponges is puzzling, because the loss of the larval stage is usually assumed to be a derived condition in invertebrates (Jägersten 1972). In addition, it is not easy to explain how the Porifera, which are currently understood as the most basal animals and should have no previous metazoan evolutionary history, have acquired several different modes of gastrulation. It is also worth recalling that the ephemeral archenteron described in the dispherula larva of homosclerophorid sponges may be interpreted as either a prelude to the archenteron in higher metazoans or a remnant of a lost gastrovascular cavity. Therefore, it cannot be discounted that other groups may have diverged earlier than sponges in metazoan history, and that the anatomy of modern adult sponges is an adaptive simplification (i.e., specialization) of the architecture of a motile larva-like ancestral poriferan, probably through acquisition of choanocytes and subsequent development of sessility and filter-feeding. If so, adult sponges are inappropriate material to provide animal homologues clarifying the transition between sponges and other metazoans. As Salvini-Plawen (1978) put it, "we are not allowed to compare the adult organization in Porifera and other Metazoa, but merely the respective larval traits."

Reinterpreting the Relationship Between Choanocytes and Choanoflagellates

If the anatomy of extant adult sponges is an adaptive simplification, perhaps by specialization of non-collared monociliated cells giving rise to choanocytes,

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feet (bf) are consistently at the posterior side of the basal body. Scale bar, 500 nm. **G.** Transverse section of a larval cilium of *S. caerulea* at the level of the plasmalemma. Note 9 anchor points (ap) surrounding the proximal end of the cilium, which consists of 9 microtubule doublets (db) and no central microtubule singlet. Scale bar, 200 nm. **H.** Longitudinal section of epithelial larval cell of *S. caerulea,* showing the proximal end of the cilium, the basal body (bb), an alar sheet (as) and an anchor point (ap), and a basal foot (bf) of complex structure, from which microtubules (m) project into the cytoplasm. Scale bar, 250 nm. **I.** Two adjacent photoreceptor cells in the posterior larval tuft of *S. caerulea,* each with a cilium and a distal, asymmetric cell protrusion filled with pigment (pg) inclusions and mitochondria (mt). Scale bar, 1 μm. **J.** Transverse section of a larval cilium of *S. caerulea* immediately above the plasmalemma, showing the 9 peripheral doublets (db) and 3 central singlets (cs). Scale bar, 100 nm.

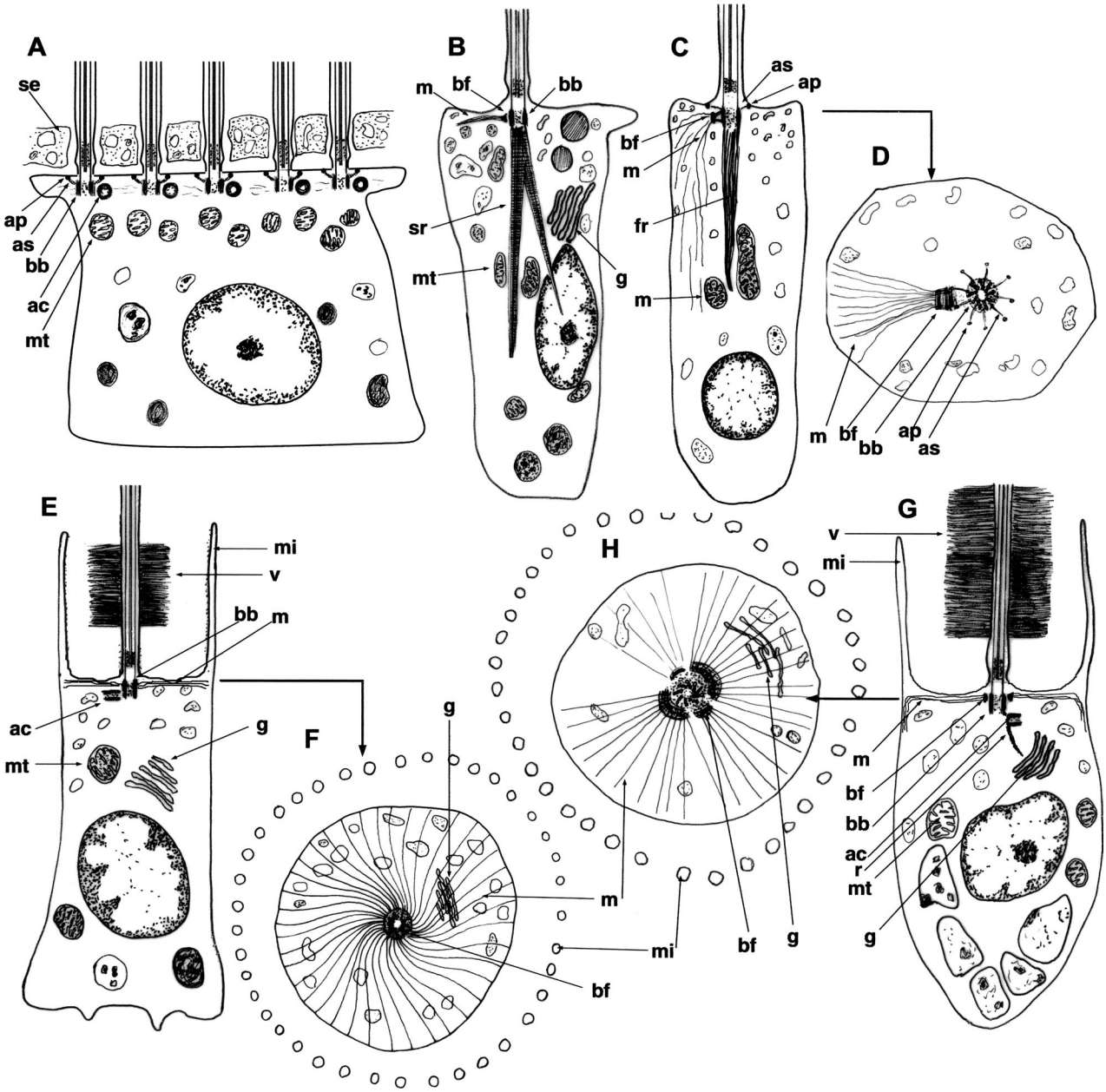

Fig. 5. Diagrams of ciliated cells of sponge larvae, choanocytes, and choanoflagellates. **A.** Subepithelial multiciliated cell of trichimella larva of a hexactinellid sponge (according to information taken from Boury-Esnault et al. 1994, 1999). **B.** Monociliated cell of calciblastula larva of a calcareous sponge (according to information from Amano & Hori 2001). **C.** Monociliated cell of parenchymella larva of a haplosclerid demosponge (information from personal observations, also from Woollacott & Pinto 1995). **D.** Transverse section of cell in C at the level of the basal foot. **E.** Choanocyte of adult sponge (according to information from Brien 1973; Simpson 1984). **F.** Transverse section of cell in E at the distal level of the basal body. **G.** Idealized choanoflagellate (according to information from Laval 1971; Hibberd 1975; Leadbeater & Morton 1974; Karpov 1982; Karpov & Leadbeater 1998). Note the fibrillar structure (r) connecting the Golgi and the accessory centriole, described only from *Monosiga ovata* and said to be a ciliary rootlet (Karpov & Leadbeater 1998). **H.** Transverse section of cell in G. Labels: accessory centriole (ac); anchor point (ap); alar sheet (as); basal body (bb); basal foot (bf); filamentous non-striated rootlet (fr); Golgi apparatus (g); microtubules (m); microvilli (mi); mitochondria (mt); putative rootlet (r) (only in *Monosiga ovata,* according to Karpov & Leadbeater 1998); syncytial epithelium (se); striated rootlet (sr); ciliary vane (v).

then the structural similarity between choanoflagellates and sponge choanocytes does not necessarily mean that choanoflagellate-like organisms were ancestors of the extant sponges and the remaining metazoans. Therefore, there are compelling reasons to re-examine the respective roles of choanoflagellates and choanocytes in the evolution of animals.

The choanoflagellates are a distinctive group of protists comprising ~150 marine and freshwater species distributed in about 50 genera and 3 main families: Codosigidae, with naked cells or thin organic investments; Salpingoecidae, with obvious periplasts or thecae; and Acanthoecidae, with a lorica of siliceous strips (Thomsen & Buck 1991). They are unicellular or colonial forms, each cell typically provided with a single cilium surrounded by a collar, a ring of retractile microvilli that contain actin filaments. The microvilli are sometimes called ''collar tentacles'' to differentiate them from microvilli that are not retractile and lack a special cytoskeleton of microtubules connected to the basal body of the cilium. The relationship of choanoflagellates to other protists is unclear, as they possess a variety of traits of uncertain affinity among protists (Corliss 1994; Patterson 1999). Most of the anatomical features that distinguish choanoflagellates also support their relationship with sponge choanocytes, particularly demosponges. Such features include not only the general cell structure and the distinctive collar, but also the secretion by some species of a skeleton of siliceous pieces similar to sponge spicules. Moreover, the symmetric vane of the cilium of choanoflagellates resembles the vane in sponge choanocytes (Fig. 5D,E), at least in the absence of studies based on more discriminating techniques than the TEM studies already conducted (Hibberd 1975; Mehl & Reiswig 1991).

In addition, choanoflagellates lack a true ciliary rootlet (e.g., Leadbeater & Morton 1974; Hibberd 1975; Karpov 1982; Leadbeater 1994), as do sponge choanocytes. In contrast, rootlets do occur in monociliated cells of sponge larvae and most metazoan monociliated cells (Fig. 5B,C). Karpov & Leadbeater (1998) reported that a short striated ciliary rootlet occurs in the choanoflagellate *Monosiga ovata* (Fig. 5G). However, unlike typical ciliary rootlets, the putative rootlet of *M. ovata* is relatively short and does not arise from the basal body, but from the accessory centriole, contacting the Golgi apparatus at the opposite end. Because of both size and position, such a structure is unlikely to be effective in rooting the cilium in the cytoplasm to palliate the mechanical tractions of beating. In addition, the number and periodicity of the cross-striated bands were not given by Karpov & Leadbeater (1998) nor are these evident from any of their published pictures. Rather, the authors described the fibrillar rootlet of *M. ovata* as reminiscent of the inconspicuous, lightly striated and asymmetrical fibrillar rootlet of heterotrophic flagellates in the genus *Phalansterium.* In contrast, cross-striation is a conspicuous feature when it occurs in ciliary rootlets of both protists and metazoans. Therefore, there are reasonable doubts that the fibrillar structure connecting the accessory centriole and the Golgi of *M. ovata* is a typical striated ciliary rootlet.

Additional similarities between choanoflagellates and sponge choanocytes are found in the ciliary basal foot and radiating microtubules. The basal body of choanoflagellates is surrounded by an electron-dense annulus or composite arc (Fig. 5E), from which microtubules radiate towards the surrounding cytoplasm (Laval 1971; Leadbeater & Morton 1974; Hibberd 1975; Karpov 1982; Leadbeater 1994). A similar structure (Fig. 5D) surrounds the basal body in demosponge choanocytes (De Saedeleer 1929; Garrone 1969; Laval 1971; Karpov & Efremova 1994) and has been interpreted as a distinctive type of basal foot (Woollacott & Pinto 1995). Some authors (e.g., Karpov & Leadbeater 1998) have reinterpreted the microtubules that radiate from the annular basal body of choanoflagellates as a ''microtubule'' ciliary rootlet, but it is indeed part of the cytoskeleton of the collar tentacle, as also appears to be the case in sponge choanocytes. Leadbeater (1994) showed that during mitosis of the choanoflagellate *Stephanoeca diplocostata,* the axonemal portion of the cilium is withdrawn deep into the cytoplasm and resorbed, but the basal body, its annular basal foot, and the associated microtubules are not. Rather, the basal body duplicates and the 2 resultant basal bodies (each provided with an annular basal foot and radiating microtubules) migrate to the opposite poles of the dividing nucleus to form the bases for the spindle of microtubules. Apparently, the radiating microtubules that surround the basal body must be maintained during mitosis because they are part of the cytoskeleton of the collar tentacles and somehow they ensure that the tentacles are shared out equitably and are moved in a coordinated manner to their new locations on the daughter cells (Leadbeater 1994). Significantly, a similar spatial relationship between the radiating microtubules and the bases of the collar tentacles may be inferred from some transverse sections of sponge choanocytes (Fig. 5F) (see Garrone 1969).

Some authors (e.g., Hibberd 1975) initially viewed choanocytes and choanoflagellates as fundamentally different, concluding (mistakenly) that the nucleus of choanoflagellates is nucleolated whereas that of choanocytes is not. It is now known that a nucleolus occurs in choanocytes of all calcareous sponges, all hex-

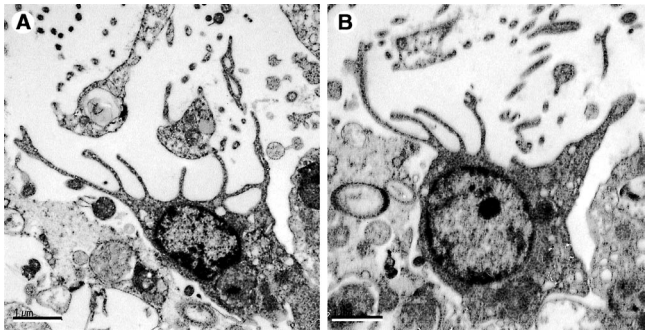

Fig. 6. Choanocytes of the demosponge *Chondrilla nucula.* TEM micrographs containing both longitudinal and transverse sections. **A.** Choanocyte showing a nucleolate nucleus. **B.** Choanocyte showing that some microvilli are either branched or engaged in particle capture. Scale bars, 1 μm.

actinellids, all homosclerophorids and some other demosponges (Fig. 6A), as well as in choanocytes of most demosponges when these cells are about to become spermatogonia (Simpson 1984). Indeed, the structural similarity between sponge choanocytes and choanoflagellates is overwhelming.

Monociliated cells bearing a collar of microvilli surrounding the cilium have been found in virtually all metazoan phyla (Nielsen 1987). These choanocyte-like or collar cells possess non-retractile microvilli, unlike sponge choanocytes (Fig. 6A,B). In these other metazoans, the cilium is often—but not always—immobile, lacks the vane, shows a striated rootlet, and in many cases is involved in sensory processes. Those cells whose cilium is mobile function in excretion (e.g., cyrtocytes of the protonephridia) or in re-circulation of coelomic fluid (e.g., in the tube feet of echinoderms), but are never involved in particle capture. For these reasons such cells are not considered homologues of the cell lineage represented by sponge choanocytes and choanoflagellates (Hibberd 1975; Salvini-Plawen 1978; Storch 1979; Nielsen 1987). Furthermore, choanocyte-like cells may derive ontogenetically from all 3 blastodermal layers, depending on the phylum (Storch 1979). In contrast, despite early doubts (Misevic et al. 1990), there is now solid evidence from Demospongiae and Calcarea that choanocytes of adult sponges derive directly from ciliated cells (usually micromeres) of the larval epithelium, which internalize during metamorphosis (e.g., Tuzet & Paris 1964; Borojevic & Lévi 1965; Amano & Hori 1998, 2001; Leys & Degnan 2002). The Hexactinellida remain uninvestigated in this regard (Boury-Esnault et al. 1999).

The ontogenetic derivation of choanocytes from non-collared ciliated cells appears to go against the idea of choanocytes as the fundamental cell type from which the remaining metazoan cells were derived. Rather, the pattern is the opposite: choanocytes are cells peculiar to sponges with no homologue among the remaining metazoans and develop from non-collared ciliated larval cells that share most of their features with other metazoan ciliated cells. The finding that choanocytes have been lost in carnivorous sponges (Vacelet & Boury-Esnault 1995) offers further support to the idea that the sponge choanocyte is a specialized cell type primarily evolved for particle capture in filter feeding. In adult sponges, choanocytes are responsible not only for capturing food, but also for producing oogonia and spermatogonia at the time of sexual reproduction (reviewed in Simpson 1984). Should a sponge under selective pressure achieve extreme histological reduction and still keep its machinery for feeding and sexual reproduction, all its cells could be lost but the choanocytes. Choanoflagellates may represent the extreme of such a reduction process, and be hyper-simplified sponge-derived metazoans rather than protists. Indeed, a lesser degree of reduction may be exemplified by in some multicellular forms, such as *Proterospongia haeckeli,* which consists of a globate colony formed by an external layer of collared cells with the cilium pointing outwards and an inner mass of unciliated amoeboid cells that divide and move in a viscous intercellular matrix apparently similar to that of sponge mesohyl (Buss 1987). Sponge choanocyte chambers that are dissociated experimentally produce globate aggregates of choanocytes with the cilia pointing outward and occasionally surrounding unciliated internal cells (Brien 1937).

The idea that choanoflagellates may be animals is not new. It was initially proposed by Nielsen in 1985, who later adopted a more conventional position, reconsidering this group within the protists (Nielsen 2001). A study by Lipscomb et al. (1998) on the origin and relationship of invertebrates using parsimony analysis for rRNA and morphological data concluded that, although the relationships between the lower invertebrates remain largely unresolved, there is sufficient morphological evidence to postulate that choanoflagellates could be sponges simplified to the unicellular level. The mitochondria of choanoflagellates, which are characterized by non-discoid flattened cristae, and their basal bodies, which bear accessory centrioles, are two features that are shared with metazoans and do not occur in other protists. Indeed, the hypothesis that choanoflagellates are reduced metazoans cannot be disproved by recent molecular approaches involving choanoflagellates, despite the goal of these studies to confirm choanoflagellates as the protist ancestors of metazoans.

For instance, several studies on genes coding for very conservative eukaryotic proteins, such as heat-

shock proteins (Hsp 70), elongation factors (EF-2), alpha-tubulin, and receptor tyrosine kinase (RTK), have provided solid, independent support to the hypothesis that choanoflagellates are more closely related than are fungi to metazoans (King & Carroll 2001; Snell et al. 2001). Such findings have been regarded as strong evidence supporting the phylogenetic relationships depicted in Fig. 1C. However, when Snell et al. (2001) tested whether choanoflagellates could be placed within Metazoa, they found that trees depicting choanoflagellates either basal to bilateralians or basal to diploblasts and sponges were equally likely as the tree in Fig. 1C and more likely than trees in Fig. 1A & B. Similarly, King & Carroll (2001) acknowledged that the idea that choanoflagellates may be derived from sponges cannot be ruled out by their finding of RTK. Nonetheless, a comparison of the mitochondrial genomes of protists and crown eukaryotes yields at least 13 genes in choanoflagellates that are absent from animal mitochondria (Gray et al. 1998), and King & Carroll (2001) concluded that ''shoehorning choanoflagellates into the Metazoa would require choanoflagellates to have re-acquired genes lost since the divergence of fungi and animals, a non-parsimonious assumption that makes the choanoflagellates-from-animals scenario highly unlikely.'' However, such a conclusion may be premature, since the complete mitochondrial genome of any sponge has yet to be published. Also noteworthy are results of a study by Ragan et al. (1996) that aimed to clarify the position of the mesomycetozoeans relative to fungi and metazoans on the basis of sequences of nuclear-encoded SSU rRNA genes. These authors found maximum-likelihood trees in which the two choanoflagellates included in the analysis (*Acanthocoepsis unguiculata* and *Diphanoeca grandis*) were placed within Metazoa.

The morphological evidence discussed here suggests that sponge choanocytes are specialized cells with no recognizable homologue in the remaining metazoans. From this perspective, in contrast to the view expressed by King & Carroll (2001), choanoflagellates are better interpreted either as reduced demosponges or as representatives of a sister group that shared with sponges a common archimetazoan (non-protist) ancestor. Indeed, such a hypothesis provides coherent accommodation for the available body of ultrastructural and molecular evidence, and also explains why there is a transitional gap in organismal architecture between choanoflagellates and the remaining protists and between adult sponges and the remaining metazoans.

The idea that the simple anatomy of extant adult sponges may be a derived condition, and that choanoflagellates may represent the extreme of such a reduction should not be shocking. Long ago Lankester (1877, 1880) suggested the possibility that some of the ''protozoans'' could have descended from multicellular animals by degeneration. Furthermore, recent findings suggest that the Myxosporidia, long considered to be a group of parasitic protozoans that produce multicellular spores, may be extremely reduced metazoans related to cnidarians, as indicated by their ability to synthesize collagen and the possession of organelles with remarkable resemblance to cnidae (Smothers et al. 1994; Siddall et al. 1995; Cavalier-Smith 1998a; Patterson 1999). I contend here that the Myxosporidia (Myxozoa) may not be the only group that experienced such an extreme simplification, but also the choanoflagellates.

Reinterpreting the Microbial Roots of Animals

Under the hypothesis that choanoflagellates may be reduced, sponge-derived metazoans, the most solid connection that scientists have so far established between protists and metazoans would apparently vanish. Nonetheless, mitochondria with flat cristae have recently been reported in diverse microbes other than choanoflagellates. Phylogenetic analyses of 18S small subunit rDNA genes place these taxa, traditionally held to be fungi, algae, or protozoans, at the boundary between fungi and animals, providing the core for a whole new phylum referred to either as Mesomycetozoa (*sensu* Mendoza et al. 2002) or as Choanozoa (*sensu* Cavalier-Smith & Chao 2003). Besides the choanoflagellates (class Choanoflagellatea), the phylum is proposed to contain 4 other classes: Mesomycetozoea, Corallochytrea, Cristidiscoidea, and Ministeriida.

The members of Mesomycetozoea are parasitic and saprotrophic microorganisms with complex life cycles, of which little is known. All species appear to go through at least 1 unciliated amoeboid or spherical cell stage, which sooner or later produces either spores or endospores in cysts, sporangia, spherules, or hypha-like structures. Many of them also produce monociliated zoospores, some develop amoeba-like cells *in vitro,* and some show a plasmodium-like structure with multiple nuclei prior to the formation of new spores. Experimental evidence indicates that some species shift between amoeba-like and hypha-like morphology in response to pH changes in the cultures. More importantly, most species—but not all—have mitochondria with flat cristae and chitin in the cell wall (Mendoza et al. 2002). Sexual fusion, gamete formation, and meiosis have so far not been recorded.

The class Corallochytrea contains a single species,

Corallochytrium limacisporum, an unciliated saprotrophic marine protist described from a coral reef lagoon (Kumar 1987). This spherical, single-celled organism undergoes successive binary fissions and becomes packed with daughter cells (up to 32), which are finally released through one or more pores in the cell wall. The daughter cells, considered endospores by some authors, behave as elongated amoeboid cells. Significantly, the mitochondria have flat cristae. However, no ciliated stages are known.

The class Cristidiscoidea comprises the nucleariid amoebas, which are free-living, freshwater cells with radiating filopodia, with one or many nuclei, and with or without a mucous envelope. They are characterized by mitochondria with both flat and discoidal cristae, but it is not clear whether these amoebas have developed discoidal cristae independently from other microbes that possess this uncommon type of crista. As in Corallochytrea, ciliated stages remain unknown in nucleariid amoebas (Zettler et al. 2001).

The Ministeriida comprises 2 species, both in the marine genus *Ministeria.* These spherical, unciliated protists are characterized by the presence of 20 symmetrically distributed, stiff, radiating pseudopodia, and mitochondria with flat cristae. One of the species, *M. vibrans,* adheres to substrates by a vibratile stalk, which appears to be a degenerate cilium (Cavalier-Smith & Chao 2003). It has also been suggested that pseudopodia of *Ministeria,* which are used to capture and ingest bacteria, may be homologues of the microvillous collar of choanoflagellates (Cavalier-Smith & Chao 2003). If so, the features of *Ministeria* further support the hypothesis that collar-bearing organisms (sponges and choanoflagellates) have been or are still immersed in an evolutionary process of morphological simplification. According to a recent study based on 18S rRNA genes, *Ministeria* groups with choanoflagellates plus *Corallochytrium,* or with choanoflagellates alone, or even more often with animals, depending on the taxon sample and phylogenetic algorithm (Cavalier-Smith & Chao 2003). In some maximum-likelihood trees using quartet puzzling with empirical base frequencies, *Ministeria* even fell within sponges, although with low statistical support. Such a tree topology, which would again be consistent with the hypothesis that choanoflagellates derive from sponges, was considered untrustworthy by Cavalier-Smith & Chao (2003). Nonetheless, it is likely that the long branch characterizing *Ministeria* in most molecular phylogenetic trees hinders any reliable placement for this taxon.

Despite recent advances, much controversy remains about the exact position of these groups at the animal-fungal divergence. Ragan et al. (1996), using sequences of nuclear-encoded ssu-rRNA genes for representatives of 3 genera of mesomycetozoeans (*Dermocystidium, Ichthyosphonus,* and *Psorospermium*), found that the position of the mesomycetozoeans in the trees changed depending on the sequences used for the phylogenetic inference. Trees produced by both parsimony and maximum-likelihood analyses of the most stably aligned sequence regions depicted the mesomycetozoeans as the most basal branch of the Metazoa. Nonetheless, within a limited range of model parameters, and in some analyses that included less well-aligned sequence regions, the trees depicted the mesomycetozoeans diverging immediately before the animal-fungal dichotomy. In contrast, phylogenetic analyses by Cavalier-Smith & Allsopp (1996), Cavalier-Smith (1998b), and Mendoza et al. (2002) find the mesomycetozoeans to be the sister group to a clade including choanoflagellates, *Corallochytrium,* and nucleariid amoebas. In the analysis by Mendoza et al. (2002), it is stressed that none of the deeper branches relating Mesomycetozoea to other clades are well supported by bootstrap values. It is also unclear whether the absence of ciliated stages in Corallochytrea and Cristidiscoidea is an ancestral or derived condition. A study by Cavalier-Smith & Chao (2003) suggests the possibility that *Corallochytrium, Nuclearia,* and Icthyosporea (1 of the 2 lineages of mesomycetozoeans) are closer to fungi than to animals. Indeed, the presence of cell walls with chitin in some classes, the absence of cilia in some groups, the co-occurrence of mitochondria with flat cristae and mitochondria with tubular, and even discoidal cristae in some groups, along with the lack of information about sexual reproduction in most cases, complicate any global comprehensive interpretation yet advanced.

Because of these contradictions and because more and more taxa seem to diverge from the so-called eukaryotic crown, some authors have cautioned about the possibility of artifacts in molecular phylogenies caused by extremely different rates of evolution. The Microsporidia, a large group of intracellular parasites long considered to be early-diverging eukaryotes because they lack the mitochondria and peroxisomes of most protozoans, may serve to illustrate the problem. Molecular phylogenies based on the large subunit of the RNA polymerase II (RPB1) gene have revealed that Microsporidia is closely related to Eumyceta (Hirt et al. 1999), probably a derived type of fungus. This recent view that Microsporidia belongs within Opisthokonta (Van de Peer et al. 2000) contradicts previous phylogenetic studies based on SSU rRNA and protein translation elongation factors (EF-1α and EF-2), which supported the early divergence of Microsporidia and included it along with other amitochondrial protists within the kingdom Archezoa. During proliferative

stages, microsporidians are unciliated, amorphous, rounded, irregular, or elongate cells, with a very simple structure and one or many nuclei within the cytoplasm. They produce spores containing a tightly coiled polar tube, by means of which the spore's nucleus and cytoplasm are introduced into a host cell following ingestion of the spore by a suitable host. Remarkably, the spores have a double-layered wall, a outer wall consisting largely of glycoprotein and an inner wall of chitin. The presence of chitin and trehalose, as well as some characteristics of the meiotic and mitotic cycles, also appear to support an evolutionary relationship between fungi and microsporidians. However, no obvious structural synapomorphies connect Microsporidia to Fungi or to any subset of fungi.

As in the case of microsporidians, if choanoflagellates are indeed extremely simplified sponges, their placement in phylogenetic trees is likely to be strongly affected by problems involving long-branch attraction. The problem may be even more severe for this group because in nearly all metazoan-focused approaches, choanoflagellates are used as the outgroup. Such a combination of circumstances may have resulted in the lack of any clade composed of choanoflagellates plus sponges in the overwhelming majority of published trees based on molecular or morphological data. Therefore, corrected phylogenetic approaches, along with tests of congruence between morphological and molecular information, are needed to elucidate the phylogenetic relationships of the choanoflagellates. More importantly, other groups, such as Corallochytrea, Cristidiscoidea, Ministeriida, and Mesomycetozoea, are emerging as a promising source of information in addition to the choanoflagellates in the search for the origin of animals.

Future Challenges

Identification of gametes and a recognizable embryonic pattern may provide the only unequivocal clue to discern between metazoan and protozoan levels. Other potential criteria, such as molecular markers and multicellularity vs. coloniality, do not provide a clear distinction in practice. Unfortunately, sexual reproduction has never been described in choanoflagellates—not a proof that it does not occur. There is a suspicion that some form of sexuality may be responsible for both the intraspecific and interspecific morphological diversity in choanoflagellates (Thomsen & Larsen 1992). The information on life-history patterns in this group, though scarce, is enough to show that choanoflagellates have complex, polymorphic life cycles involving several cell types and alternation of unicellular and colonial stages, as well as sedentary and dispersing phases (Leadbeater 1977, 1983). Several choanoflagellates with a sessile trophic stage also produce free-swimming cells, interpreted as zoospores. They are notably smaller, consistently naked, have no collar, and swim vigorously with the cilium propelling the cell from behind, just as in animal sperms. However, it remains unresolved whether they are sexual cells, because the diploid or haploid condition of the various stages in choanoflagellate life cycles remains uninvestigated.

Some of the evidence reviewed here suggests that the search for potential metazoan ancestors should be re-directed, or at least extended, to include non-collared protists, particularly those sharing cytological structures or molecules with sponge larvae rather than adult sponges. In this regard, the occurrence of multiciliated and several types of multinucleate cells in larvae of hexactinellid sponges does not allow us to rule out multicilated and multinucleate protist lineages as putative metazoan ancestors. The panoply of morphological possibilities for both the putative archimetazoan and the putative poriferan descendent can be reliably reduced by first resolving the phylogenetic relationships within Porifera. The obscure relationships within Porifera, as well as between Porifera and the remaining diploblastic phyla, could be further clarified by molecular approaches considering not only information from adults, but rather genes and proteins operating in embryonic and larval sponge cells, an area of research virtually neglected so far. The diversity and complexity of some developmental traits suggest the possibility that other groups of organisms diverged earlier than the sponges in metazoan history. If so, it remains unclear what kind of organisms they were, but such a possibility should be kept in mind when discussing and re-interpreting known and new palaeontological evidence.

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