Adult Stem Cells Host Intracellular Symbionts: The Poriferan Archetype

Alexander Ereskovsky, Baruch Rinkevich and Ildiko M. L. Somorjai

Abstract: Unlike vertebrates, adult stem cells (ASC) in a wide range of aquatic invertebrate phyla are morphologically diverse, exhibiting a wide range of differentiation states as well as somatic and germline physiognomies. They may arise de novo by trans-differentiation from somatic cells and above all represent phenotypes of specialized cells with multifunctionality. One unexpected phenomenon is the presence of intracellular symbionts in the ASCs of some invertebrates. Overviewing the literature on intracellular symbionts in sponge (Porifera) ASCs and in other aquatic invertebrates, we reveal that ASC intracellular prokaryotic and eukaryotic symbionts are restrictive to a single sponge class, the Demospongiae. The eukaryotic symbionts in sponges are exclusively unicellular photosynthetic algae, and are found only in pluripotent stem cells, most frequently in the archaeocytes; they are documented in five orders of Demospongiae. Bacteriocyte-like cells have been reported in sponges and three other phyla, indicative of their independent evolutionary origins. The results of this study add considerable insight into the establishment and maintenance of intracellular symbioses in ASCs of aquatic invertebrates, and provide new a understanding of the diversity of symbiotic associations across the tree of life.

1. Introduction

According to the prevailing dogma in cell biology, adult stem cells (ASC) in animals are committed lineage-specific cells, with tissue-/organ-restricted fates, and which are moreover capable of regeneration and repair of tissues and organs (Clevers and Watt 2018). Ordinarily, ASCs are undifferentiated cells that give rise to either daughter stem cells, non-self-renewing progenitors, or to lineage-specific differentiated cells (Clevers and Watt 2018; Raff 2003). Model ASCs (in vertebrates and insects) typically possess high nucleo-cytoplasmic ratios, are small in size compared to lineage-differentiated progenies, and are often rare. However, ASCs in many aquatic invertebrates are not only very common (up to one third of all animal cells), but are also morphologically highly diverse, and exhibit a wide range of differentiation states as well as somatic and germline characteristics, just to name some key biological properties (summarized in Rinkevich et al. 2022). Moreover, ASCs in aquatic invertebrates may arise de novo by trans-differentiation from somatic cells (Borisenko et al. 2015; Ferrario et al. 2020) and above all represent phenotypes of specialized cells with multifunctionality. Examples include the ecto-/endodermal epitheliomuscular cells in polyps of Cnidaria (Bosch et al. 2010; Hobmayer et al. 2012) or the archaeocytes and choanocytes in Porifera (Funayama 2018).

One unexpected and, as yet, little-explored phenomenon is the presence of intracellular symbionts in the ASCs of some invertebrates (for example, Bright and Giere 2005; Masuda 1990; Pflugfelder et al. 2009; Saller 1989), and the evidence that ASCs manipulate symbiont maintenance (Bosch et al. 2010; Dirks et al. 2012; Kovacevic 2012). Below, we review the literature on ASCs and their symbionts in sponges (Phylum: Porifera), which represent the best-known model case, as well as the few examples from other systems. We place this within the context of intracellular symbionts more generally, concluding with a discussion of how the application of modern methodologies in sponges to this problem may improve our understanding of this unusual symbiosis.

2. Symbiosis

2.1. What Is Endosymbiosis?

Symbiosis, an inter-dependent relationship between two species, is an important factor for ecological diversity and evolutionary novelty (Sitte and Eschbach 1992; Wernegreen 2012). The most comprehensive definition of symbiosis includes the full range of interaction modes, from harmful (parasitic) to beneficial (mutualistic). It applies not only to organisms living anywhere within the host body—such as within tissues (extracellular) or within cells (intracellular)—but also to cytosymbiosis, the intimate and long-lasting association of cells belonging to different taxa, and often considered as the most intricate partnership among living entities (Sitte and Eschbach 1992; Wernegreen 2012). Both parasitic and mutualistic symbiotic interactions can evolve into a state where there is a stable and permanent association between symbionts and hosts. In the case of intracellular mutualists, evolutionary processes may lead to cytosymbiosis through both morphological alterations as well as via physiological/molecular incorporation of the symbionts into the hosts' cellular environments, to the point where endosymbionts are no longer easily recognizable as foreign intrusions. Following such integrations, endosymbionts enhance the ability of hosts to succeed in diverse contexts, from unbalanced diets and nitrogen-poor soils, to hydrothermal vents and oligotrophic aquatic environments (Hinzke et al. 2021; Wernegreen 2012). Key functions performed by mutualistic, intracellular endosymbionts include harvesting energy from chemicals or light, to converting nitrogen into a usable form, and synthesizing nutrients that supplement the host's diet, to name just a few (Wernegreen 2012).

Cytosymbiotic associations can be organized within a graded series of cumulative morphological integrations, including the development of arrays of mechanisms targeting the interactions between host and symbiont (Bandi et al. 1995; Melo-Clavijo et al. 2018; Song et al. 2017). They can also be exposed to partner switching and rapid compensatory evolution (Sørensen et al. 2021). In cytosymbiosis, the interrelations between the partners of each specific symbiosis can be commensalic, parasitic, or mutualistic; but in every case, cytosymbiotic partnership leads to adaptive interaction of the partners or even to strict co-evolution (Sitte and Eschbach 1992). Intracellular symbiotic microorganisms commonly reside in specialized or non-specialized host cells, but not in ASCs; the property of "stemness" could be regarded as mutually exclusive to a highly differentiated and specialized cytosymbiotic state. The appearance of such an association, therefore, should be studied not only from functional but also from host/symbiont co-evolutionary perspectives, as unicellular symbionts have been associated with sponges (and their ASCs) since their initial evolution as multicellular animals (Ereskovsky 2010; Wilkinson 1983).

2.2. Porifera as Model Systems for ASC Cytosymbiosis

2.2.1. Overview of Characteristics of Organization and Cellular Plasticity

Sponges branch off basally in the metazoan phylogenetic tree and comprise four distinct classes: Demospongiae, Hexactinellida, Calcarea and Homoscleromorpha. Living sponges are found in all aquatic environments at all depths. A sponge is traditionally defined as "a sedentary, filter-feeding metazoan", and has no nerves, muscles, specialised digestive system or gonads (Borchiellini et al. 2021).

Sponges have two cell layers, the choanoderm and the pinacoderm (Figure 1), formed by choanocytes and pinacocytes, respectively. Choanocytes are flagellated collar cells lining the filtering cavities of the aquiferous system, the choanocyte chambers. Pinacocytes are flattened cells covering the outer parts of the body and lining the canals of the aquiferous system. The space between the external pinacocyte layer and the aquiferous system is filled by the mesohyl, a loose layer composed of collagen fibrils, skeletal elements, and up to ten cell types with different degrees of motility (Ereskovsky and Lavrov 2021; Harrison and De Vos 1991).

The tissues in sponges are simpler, both structurally and functionally, than in other Metazoa. In particular, sponge tissues tend to be highly multifunctional when compared to counterparts in more recent branching animal lineages, permitting a higher rate of cell migration and thus an almost constant reorganization of tissues. Moreover, the cells of sponge tissues possess a very high capacity for transdifferentiation into other cell types (Gaino et al. 1995; Nakanishi et al. 2014). In addition, sponges possess very high regenerative and reconstitutive abilities, culminating in the re-building of a functional body from dissociated cells (reviewed in Ereskovsky et al. 2015, 2020, 2021; Lavrov and Kosevich 2014; Simpson 1984).

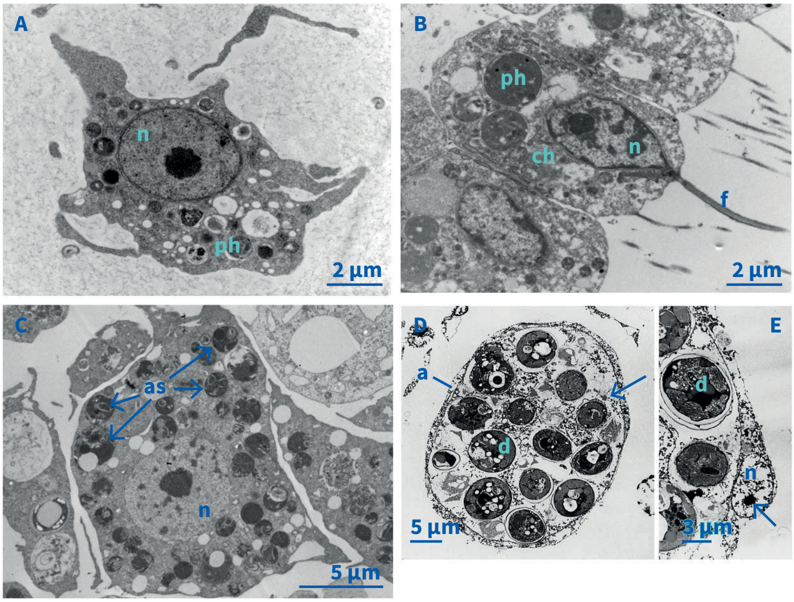

Figure 1. TEM images of principal pluripotent cells in sponges. (**A**)—archaeocyte of *Halisarca dujardinii* (Demospongiae); (**B**)—choanocytes of *Leucosolenia variabilis* (Calcarea); (**C**)—archaeocyte of the freshwater sponge *Lubomirskia baicalensis* (Demospongiae) with intra-cellular algal symbionts; (**D**,**E**)—archaeocytes of the marine sponge *Haliclona* sp. (Demospongiae) with intra-cellular dinoflagellate symbionts (showing fibrillar material between algae and archaeocyte (arrowed) (Modified from Garson et al. 1998). as—algal symbionts, ch—choanocyte, d—dinoflagellate, f—flagellum, n—nucleus, ph—phagosome. Source: Graphic by authors.

2.2.2. Sponge ASC Characteristics

As one of the most basal metazoan groups (Redmond and McLysaght 2021; Simion et al. 2017), sponges hold a key position to address stem cell origins.

Most research on stem cells in sponges has been conducted in demosponges, and until recently, consisted almost entirely of microscopic studies. However, in the past few years, molecular studies have provided new insights. According to the most recent investigations in Porifera, there are not only two (Funayama 2018), but rather at least four types of pluripotent ASC: the archaeocytes and choanocytes, as well as pinacocytes and particular amoeboid vacuolar cells (Ereskovsky et al. 2015; Fierro-Constaín et al. 2017; Lavrov et al. 2018).

Three main criteria are generally accepted as defining a stem cell: (1) the capacity for self-renewal, (2) differentiation (or transdifferentiation) of this cell type into others, and (3) contribution of this cell to the processes of homeostasis and regeneration (Melton 2014). The molecular evidence of their stemness includes the expression of genes encoding GMP (germline multipotency program) proteins (*piwi*, *vasa*, *bruno*, *pl-10*, and all the genes encoding Tudor domains, *ddx6*, and *mago-nashi*); the observation that genes encoding RNA helicase and proteins involved in mRNA splicing are elevated in the archaeocytes of the freshwater demosponge *Ephydatia fluviatilis* (Alié et al. 2015); and expression of the *EfPiwiA* and *EfPiwiB* genes detected in choanocytes (Funayama et al. 2010). GMP genes (*piwi*, *argonaute*, *vasa*, *nanos*, *pl10*, *tudor*, *pumillo*, *boule*) are expressed in the choanocytes of adult *Oscarella lobularis* (Homoscleromorpha) (Fierro-Constaín et al. 2017). *VasaB* and *SciPL10B* are also strongly expressed in the choanocytes of *Sycon ciliatum* (Calcarea) (Leininger et al. 2014).

Choanocytes are specialized epithelial cells responsible for water movement inside the sponge aquiferous system and food particle capture. These cells are characterized by apical–basal polarity and the presence of a flagellum surrounded by the collar of microvilli at the apical pole (Simpson 1984) (Figure 1B).

Archaeocytes are amoeboid cells of the mesohyl devoid of any polarity or specialized features, and are typical in Demospongiae. These cells manifest high polymorphism and multifunctionality. Up to now, no generally accepted characteristics of archaeocytes have been defined. Only general features exist, which are present in all archaeocyte descriptions: an amoeboid shape, a large nucleolated nucleus and the absence of specialized inclusions in the cytoplasm (Ereskovsky and Lavrov 2021; Simpson 1984), (Figure 1A,C). As for the function of demosponge archaeocytes, their role has been described in: (1) the transport of food particles and elimination of digestive products (Godefroy et al. 2019; Willenz and Van de Vyver 1984); (2) outgoing particulate organic matter (Maldonado 2016); (3) the burrowing processes in excavating sponges (Rützler and Rieger 1973); (4) spicules secretion (Funayama et al. 2005; Rozenfeld 1980); (5) immunity role (Fernàndez-Busquets 2008; Smith and Hildemann 1986); (6) gametogenesis (Ereskovsky 2010; Simpson 1984); (7) asexual reproduction (budding, gemmulogenesis, reduction body formation) (Ereskovsky et al. 2017; Harrison et al. 1975; Simpson 1984); (8) regeneration, somatic embryogenesis and growth (Buscema et al. 1980; Ereskovsky et al. 2020, 2021; Lavrov and Kosevich 2014). Thus, this sponge archaeocyte multifunctionality is unusual for the stem cells of Metazoa.

Notably, there is another unusual feature of archaeocytes in Demospongiae—the presence of intracellular photosynthetic algal symbionts. Freshwater sponges (order Spongillida) harbour Chlorophyta from the classes Trebouxiophyceae and Chlorophyceae (zoochlorella), and Ochrophyta from the class Eustigmatophytacea. Some marine demosponges (orders Haplosclerida and Clionaida) also harbour Dinoflagellata *Symbiodinium* spp (zooxanthella) (Table 1).

Table 1. Distribution of symbiotic intracellular unicellular algae in demosponge adult stem cells.

Demosponge Species	Adult Sponge Cell Type	Gemmule	Buds	Algal Species	Method	References
Order Spongillida						
Family Spongillidae						
Spongilla lacustris (geen)	Archaeocytes, choanocytes (Williamson)	Thesocytes of green gemmules	No	Phylum Chlorophyta Trebouxiophyceae, zoochlorellae	TEM	(Masuda 1990; Gilbert and Allen 1973; Williamson 1979)
Spongilla lacustris	Archaeocytes, choanocytes, pinacocytes	Thesocytes	No	Trebouxiophyceae, *Chlorella sp.* Germany	In vivo microscopy LM, TEM	(Saller 1989, 1991)
Spongilla lacustris	?	Thesocytes	No	Trebouxiophyceae, *Choricystis minor* - Japon	LM,	(Handa et al. 2006)
Spongilla lacustris	?	?	No	Trebouxiophyceae, *Lewiniosphaera symbiontica* USA	MB	(Pröschold et al. 2010)
Nudospongilla moorei	Amoebocytes	?	?	Zoochlorella	LM	(Brien and Govaert-Mallebranche 1958)
Radiospongilla sendai (geen)	Archaeocytes	Thesocytes of green gemmules	No	Trebouxiophyceae, (zoochlorellae) *Choricystis minor*	TEM	(Masuda 1990; Handa et al. 2006; Okuda et al. 2002)
Radiospongilla cerebellata (geen)	Archaeocytes	Thesocytes of green gemmules	No	Trebouxiophyceae, (zoochlorellae) *Choricystis minor* and *Chlorella*	TEM	(Handa et al. 2006; Masuda 1985, 1990)
Radiospongilla cerebellata	Archaeocytes, amoebocytes, choanocytes, pinacocytes	?	Archaeocytes, amoebocytes, choanocytes, pinacocytes	Trebouxiophyceae, *Chlorella*	LM, TEM	(Saller 1990)
Eunapius fragilis	?	Thesocytes	No	Trebouxiophyceae, *Choricystis minor*	LM, *In vivo* microscopy	(Handa et al. 2006)
Heteromeyenia slepanowii (geen)	Archaeocytes	No	No	Zoochlorellae	TEM	(Masuda 1990)
Ephydatia fluviatilis (geen)	Archaeocytes	Thesocytes	No	Trebouxiophyceae, *Chlorella* sp	LM, TEM	(Wilkinson 1980; Gaino et al. 2003)
Ephydatia fluviatilis (brown)	Archaeocytes	No	No	No algae	LM, TEM	(Gaino et al. 2003)
Ephydatia muelleri	Archaeocytes	Thesocytes	No	*Chlorella* sp.	LM, cell fractioning	(Hall et al. 2021)
Ephydatia muelleri	Archaeocytes	No	No	Trebouxiophyceae Choricystis, *Chlorella* sp.	MB, CM, TEM	(Masuda 1990; Gilbert and Allen 1973; Williamson 1979)

Table 1. *Cont.*

Demosponge Species	Adult Sponge Cell Type	Gemmule	Buds	Algal Species	Method	References
			Family Lubomirskiidae			
Lubomirskia baicalensis	Archaeocytes	No	No	Chlorophyceae *Mychonastes jurisii*	LM, TEM, SEM, MB	(Chernogor et al. 2013)
Lubomirskia baicalensis	Archaeocytes	No	No	Trebouxiophyceae Chlorophyceae *Mychonastes* sp.	TEM	(Ereskovsky et al. 2016)
Lubomirskia incrustans	Archaeocytes	No	No	Trebouxiophyceae *Choricystis parasitica*	LM	(Kulakova et al. 2014)
Lubomirskia abietina	Archaeocytes	No	No	Trebouxiophyceae *Choricystis krienitzii*	LM	(Kulakova et al. 2020)
Baikalospongia bacillifera	Archaeocytes	No	No	Trebouxiophyceae *Choricystis parasitica*	LM	(Kulakova et al. 2014)
Baikalospongia intermedia	Archaeocytes	No	No	Trebouxiophyceae *Choricystis krienitzii*	LM	(Kulakova et al. 2020)
			Family Metaniidae			
Corvomeyenia everetti	Archaeocytes	No	No	Phylum Ochrophyta Eustigmatophytacea	TEM	(Frost et al. 1997)
			Order Haplosclerida			
Haliclona sp.	Archaeocytes	No	No	Dinoflagellata, *Symbiodinium microadriaticum*	LM, TEM	(Garson et al. 1998)
			Order Clionaida			
Cliona viridis	Archaeocytes	?	Archaeocytes	Dinoflagellata *Symbiodinium*	LM, TEM	(Rosell 1993)
Cliona inconstans, C. orientalis	Archaeocytes	?	?	Zooxantellae	LM, TEM	(Vacelet 1981)
Cliona caribbaea, C. varians	Archaeocytes	?	?	Dinoflagellata *Gymnodinium microadriaticum*	LM, TEM	(Rützler 1990)
Cervicornia cuspidifera	Amoeboid cells	No	No	Dinoflagellata *Symbiodinium microadriaticum*	LM	(Rützler and Rieger 1973)
			Order Suberitida			
Suberites aurantiacus	Archaeocytes	No	No	Zooxantella	LM	(Cheng et al. 1968)
			Order Tetractinellida			
Cinachyra tarentina	Amoeboid cells	No	No	Dinoflagellata Zooxantella *Symbiodinium microadriaticum*	LM, TEM	(Scalera-Liaci et al. 1999)

CM—confocal microscopy; LM—light microscopy; MB—molecular biological data; no—absence; SEM—scanning electron microscopy; TEM—transmission electron microscopy; ?—no data.

2.2.3. Diversity of Intracellular Algal Symbionts

Intracellular algal symbionts were described for the first time by Brandt (1881, 1882—see Krueger 2016) in mesohylar cells of the freshwater demosponge *Spongilla* sp. Subsequently, thanks to progress in light and electron microscopy, intracellular algal symbionts were found in a number of different sponge species, but exclusively from the class Demospongiae (Rützler 1990; Sarà and Vacelet 1973; Sarà et al. 1998; Simpson 1984; Vacelet 1981; Wilkinson 1987). These symbionts include different species of the phylum Chlorophyta, the classes Trebouxiophyceae (genera *Chlorella, Zoochlorella, Choricystis, Lewiniosphaera)*, Chlorophyceae *(Mychonastes)* (Masuda 1985, 1990; Pröschold and Darienko 2020; Saller 1990; Simpson 1984; Williamson 1979), dinoflagellates (Zooxanthellae) of the genera *Symbiodinium* and *Gymnodinium* (Annenkova et al. 2011; Garson et al. 1998; Hill 1996; Pang 1973; Rosell and Uriz 1992; Rützler 1990; Sarà and Liaci 1964; Scalera-Liaci et al. 1999; Vacelet 1981), cryptophytes, cryptomonads (Wilkinson 1992), diatoms (Cox and Larkum 1983), coccoid red algae (Lemloh et al. 2009) eustigmatophytes (Frost et al. 1997), and macroscopic algae (Price et al. 1984; Rützler 1990) (Table 1).

2.2.4. Distribution of Archaeocytes with/without Symbionts in the Sponge

Archaeocytes are the principal cells acing as hosts (Table 1), and the same archaeocyte can contain from one to several algal symbionts (Gaino et al. 2003; Masuda 1990; Saller 1989). In some freshwater sponges, green algal symbionts can also be found inside choanocytes and pinacocytes (Gilbert and Allen 1973; Saller 1990, 1991). This is also true for some marine demosponges. In *Haliclona* sp., algal cells of *Symbiodinium microadriaticum* are grouped together in clusters of 6 ± 10 cells and enclosed by sponge cells, rather than being randomly distributed throughout the mesohyl (Garson et al. 1998). In the boring sponges *Cliona inconstans* and *C. orientalis*, the Zooxanthellae are always intracellular and occur in individual vacuoles of archaeocytes (Figure 1D,E). Each cell contains several algae (Vacelet 1981). In *Cliona caribbaea* and *C. varians*, the symbiotic dinoflagellates *Gymnodinium microadriaticum* are intracellular, either fully embedded in a host archaeocyte vacuole or encircled by host cell filopodia (Rützler 1990).

The spatial distribution of cells harboring symbionts in the sponge body is not homogeneous. In *Cinachyra tarentina,* the majority of the zoochlorellae are concentrated in the cortical zone of the sponge (Scalera-Liaci et al. 1999). Archaeocytes of *Ephydatia fluviatilis* harbour *Chlorella* concentrated mainly in the uppermost regions of the sponge body; in the inner parts of the sponge body, cells do not host zoochlorellae (Gaino et al. 2003).

The intracellular position of algal cells occurs in the host cytoplasm within vacuoles. At least in more thoroughly studied systems such as protists, the cnidarian *Hydra viridis* and the sponge *Spongilla lacustris*, two types of vacuole are observed (Reisser and Wiessner 1984). The first, the perialgal vacuole, always harbours only one algal cell. The wall of this type of vacuole is attached to the vacuolar membrane of the host. A perialgal vacuole divides simultaneously with the enclosed alga and apparently protects it from host lytic enzyme action (Reisser and Wiessner 1984). The chlorellae are able to divide inside the perialgal vacuole of sponge cells in *Spongilla lacustris* (Saller 1990). The second, the food vacuole, contains algae in various stages of digestion and other material (Simpson 1984). This may allow the host cell to absorb nutrients from damaged or dying algae, or under particularly adverse conditions in which the symbiont can no longer be maintained.

2.2.5. Intracellular Symbiosis Is Facultative

Three lines of evidence indicate that intracellular symbiosis of algal and sponge cells is facultative: (1) geographic, (2) ecological and (3) ontogenetic. The best geographic evidence comes from *Spongilla lacustris.* These sponges are able to host different algae species in their archaeocytes, depending on the geographic region they inhabit: *Chlorella* sp. in Germany (Saller 1989), *Choricystis minor* in Japan (Handa et al. 2006), *Choricystis parasitica* and *Lewiniosphaera symbiontica* in Massachusetts (USA) (Pröschold et al. 2010). (2) Ecological evidence. With respect to ecological evidence, many populations of the same freshwater sponge species contain green, brownish, and white individuals as a result of temporal and/or spatial variation in light availability. Electron microscopy investigation revealed that green sponges harbour zoochlorellae, which absent in the brownish ones (Gaino et al. 2003). Sponges that have green colour with zoochlorellae will quickly turn white when shaded (Frost and Williamson 1980), as zoochlorellae were digested by their host (Williamson 1979). Other examples are fresh-water sponge species that live in dark habitats, such as underground caves (*Eunapius subterraneus* in Croatia (Bilandija et al. 2007); *Racekiela cavernicola* in Brasil (Volkmer-Ribeiro et al. 2010)), or at great depths in lakes that completely lack symbiotic eukaryotic algae (e.g., *Baikalospongia abyssalis* in Baikal (Itskovich et al. 2017)).

There is also experimental evidence. For example, Hall et al. (2021) infected young aposymbiotic sponges of *Ephydatia muelleri* that had hatched from gemmules with sponge-derived algae. Evidence of the establishment of intracellular position by the algae was manifested within 4 h of infection. At the 24-hour time point, many sponge host archaeocytes harboured multiple or single algae within a single cell.

In adult sponges the algae are transmitted among the sponge cells in a very particular way. After the donor and the recipient cell getting closer each another, the vacuole includes *Chlorella* inside bulges out, surrounded by cell processes of the recipient cell. The vacuole opens, while the donor cell retracts and the recipient cell closes around the alga. Finally, the alga is incorporated into the recipient cell (Masuda 1990; Saller 1991). No release of the algae into the intercellular mesenchyme was detected. Then, the chlorella cells divide inside the sponge cells.

2.2.6. Horizontal and Vertical Transmission of Intracellular Algal Symbionts

As we showed above, the sponge-algal symbiosis is facultative. Accordingly, the transmission of algal symbionts occurs horizontally during sexual reproduction. In any event, not a single study has so far shown the presence of algal symbionts in sponge larvae. As for asexual reproduction, the situation there is more complicated.

In sponges there are three types of asexual reproduction: fragmentation, budding and gemmule formation (Ereskovsky 2010).

During fragmentation, the sponge is divided into two or more parts, each consisting of all tissue types and the symbionts. In contrast, during the budding process, the vertical transmission of intracellular symbiotic algae has been documented for two species: in the fresh-water sponge *Radiospongilla cerebellata*, where bud cell archaeocytes, amoebocytes, choanocytes and pinacocytes included *Chlorella* sp. (Saller 1990); and in the marine boring sponge *Cliona viridis*, in which the archaeocytes of the buds harbour intracellular dinoflagellate symbionts (Rosell 1993).

Gemmules of demosponges are special dormant structures that are capable, under suitable conditions, of developing asexually into new adult animals (Simpson 1984). Gemmules develop from the pluripotent archaeocytes. Gemmule thesocytes (resulting from archaeocyte differentiation) of many freshwater sponges include four or five functional algal endosymbionts per cell (Gilbert and Allen 1973; Masuda 1990; Okuda et al. 2002; Williamson 1979). The ultrastructure of zoochlorellae inside of gemmules differs from the ultrastructure of active symbionts in adult, green sponges: the gemmular symbionts contain loosely packed membranes of the chloroplasts, they generally lack lipid granules, and they lack chloroplast starch grains (Masuda 1990; Williamson 1979). This modification in structure could be a result of the relative inactivity of the symbionts inside gemmules. However, it has been shown that the symbionts within thesocytes are photosynthetically active, and could pass some of their photosynthate to the sponge cells (Gilbert and Allen 1973).

Before hatching, symbiotic algae could be phagocyted, and thus the young sponges that develop from such gemmules would be aposymbiotic (Rasmont 1970), without signs of symbiotic algal propagation (Simpson 1984; Williamson 1979). Yet, under dark conditions, brown gemmules do not host symbiotic algae, or only possess them in very low numbers (Gilbert and Allen 1973; Jorgensen 1947; Simpson 1984). Therefore, the vertical transmission of intracellular algal symbionts during gemmulogenesis in sponges is facultative.

2.3. Cytosymbiosis in ASCs-Beyond Poriferans

In contrast to the demosponges, cytosymbiosis in ASCs is a rare situation in other multicellular organisms in general, and in marine invertebrates in particular (Figure 2). Nevertheless, several well documented cases attest to the importance of ASCs in coordinating and maintaining intracellular symbiosis. Examples include the deep-sea vestimentiferan tubeworms (Polychaeta; best known are *Lamellibrachia luymesi* and *Riftia pachyptila*), which live in symbiosis with intracellular bacteria housed in bacteriocyte host cells (considered to be "tissue-specific unipotent bacteriocyte stem cells"; (Pflugfelder et al. 2009)), located within a special organ, the trophosome (Bright and Giere 2005). These stem cells continuously proliferate to produce new bacteriocytes, a process leading to self-renewal of bacteriocyte and to a complex control of the symbiont population in these host cells. Similarly, the free-living symbiotic flatworm *Paracatenula galateia* possesses intracellular, sulphur-oxidizing bacteria (also called bacteriocytes): as for all other somatic cells in adult worms, the bacteriocytes originate solely from the pool of aposymbiotic neoblasts, the ASCs of flatworms (Dirks et al. 2012). In addition, in *Hydra*, the epithelial stem cells lineages, but not the interstitial cells, actively shape the microbial intracellular communities of epithelial cells (Fraune et al. 2009). However, the elimination of nerve cells and secretory gland cells, two important cell types derived from interstitial cells, had a significant influence on the structure of symbiotic microbiota. Further, in the branching coral species *Stylophora pistillata*, algal containing cells in the endodermal layer express "stemness" genes such as *Nanos* and *Tudor*, as well as Tubulins and genes involved in the cell cycle (Levy et al. 2021), indicating that these cells may carry stem cell properties. There is also some preliminary evidence for ASC-related cytosymbiosis in hibernating colonies of botryllid ascidians (Hyams et al. 2017). About 15% of the blood cell population in the vasculature of hibernating colonies was first identified as phagocytes. However, transmission electron microscope studies revealed specific facultative symbionts—*Endozoicomonas* bacteria—inside their phagosomes. This novel case of cytosymbiosis develops de novo and only during stress conditions, a phenomenon most probably controlled by circulating ASCs (B.R., unpublished data).

There are additional unique examples of the involvement of ASCs of terrestrial invertebrates in maintaining or controlling intracellular symbionts. In early developmental stages of the aphids Acyrthosiphon pisum and Megoura viciae and in the cockroach Periplaneta americana, studies revealed de novo bacteriocyte formation from aposymbiotic ASCs, followed in the cockroaches by postembryonic divisions of the bacteriocytes (Braendle et al. 2003; Chevalier et al. 2011; Lambiase et al. 1997; Maire et al. 2020; Miura et al. 2003), suggesting that insect and tubeworm bacteriocytes proliferate (Dirks et al. 2012). The same applies to haemocytes of the isopod Armadillidium vulgare, which host endosymbiotic Wolbachia cells, intracellular α-proteobacteria (Chevalier et al. 2011) that are considered parasites in many insects such as Drosophila, in which they colonize female germline stem cells (Ote and Yamamoto 2020). However, some strains also appear to confer protection against RNA viruses in flies and mosquitoes in the laboratory, indicating a mutualism, although it is still unclear if this antiviral effect exists in the wild (reviewed in (Pimentel et al. 2021)). Intracellular Wolbachia symbionts are not only the cytosymbiotic bacteria in insect stem cells, as germline cells can also be colonized by other microorganisms, such as the Gram-positive bacterium Spiroplasma in Drosophila (Hackett et al. 1986), or the Gram-negative bacterium Arsenophonus, which infects the Sulcia symbiont of the leafhopper Macrosteles laevis (Kobiałka et al. 2016). The aforementioned means of ASC control is further illustrated by bacteria from larval bacteriocytes in uninfected nuclei of putative stem cells, as assessed over the course of metamorphosis (Maire et al. 2020).

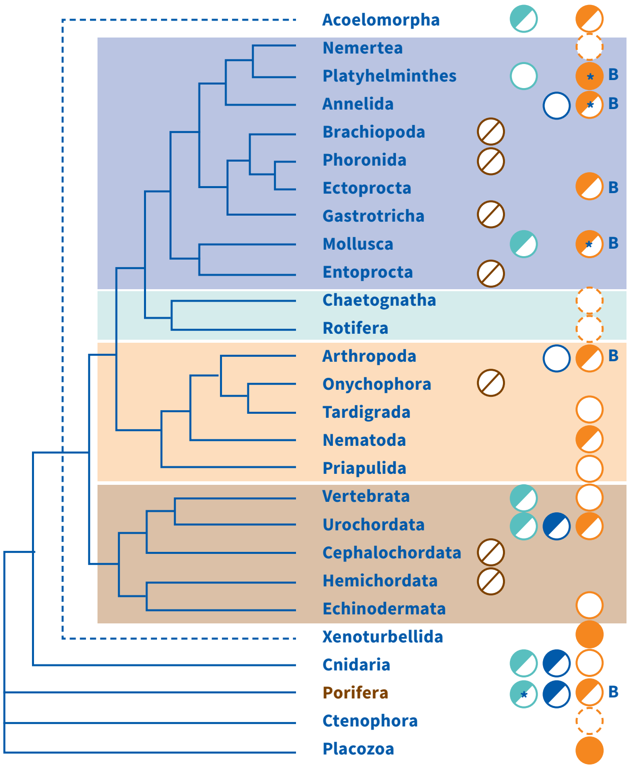

Figure 2. Distribution of algal, cyanobacterial and bacterial endosymbionts in metazoan phyla. To the left, a cladogram illustrates phylogenetic relationships among phyla; branch lengths are not proportional to evolutionary divergence. The position of sponges (Porifera) is highlighted in red. Coloured boxes indicate bilaterian lineages belonging to the Ecdysozoa (yellow), Gnathifera (green) Lophotrochozoa (blue), and Deuterostomia (pink). The positions of Acoelomorpha and Xenoturbellida are still debated and are indicated by dotted lines. To the right of each phylum, absence of endosymbionts (red symbols) as well as presence of algal (green symbols), cyanobacterial (blue symbols) and bacterial (orange symbols) endosymbionts are shown. Note that for many lineages, examples of both (mixed circles) intracellular (filled circles) and extracellular (empty circles) symbionts exist and where endosymbiosis is uncertain, dotted circles are used. Groups in which bacteriocytes have been reported are indicated by a "B" next to the bacterial endosymbiont column. An asterisk (*) denotes cases in which ASCs have been reported in the literature to contain endosymbionts. Sources: Acoelomorpha:

(Melo-Clavijo et al. 2018; Hikosaka-Katayama et al. 2012; Venn et al. 2008); Nemertea: (McDermott 2006); Platyhelminthes: (Dirks et al. 2012; Dubilier et al. 2008; Gruber-Vodicka et al. 2011; Melo-Clavijo et al. 2018; Venn et al. 2008); Annelida: (Dubilier et al. 2008); Ectoprocta: (Karagodina et al. 2018; Saffo 1992; Sharp et al. 2007); Gastrotricha: (Todaro et al. 2017); Mollusca: (Dubilier et al. 2008; Duperron et al. 2006; Melo-Clavijo et al. 2018; Venn et al. 2008); Chaetognatha: (Thuesen and Kogure 1989); Rotifera: (Selmi 2001); Arthropoda: (Dubilier et al. 2008; Lindquist et al. 2005); Tardigrada: (Vecchi et al. 2016); Nematoda: (Dubilier et al. 2008); Priapulida: (Kroer et al. 2016); Vertebrata: (Baker et al. 2019; Kerney et al. 2011; Melo-Clavijo et al. 2018); Urochordata: (Melo-Clavijo et al. 2018; Mutalipassi et al. 2021; Saffo 1992); Echinodermata: (Carrier and Reitzel 2020; Saffo 1992); Xenoturbellida: (Kjeldsen et al. 2010); Cnidaria: (Melo-Clavijo et al. 2018; Venn et al. 2008); Porifera: (Rützler 1990; Saller 1991; Sarà et al. 1998; Williamson 1979); Ctenophora: (Daniels and Breitbart 2012; Hernandez and Ryan 2018); Placozoa: (Gruber-Vodicka et al. 2019). Please see text for details.

The important interplay between ASCs and their intracellular symbionts has also been recorded in vertebrates. For instance, the intracellular bacterial pathogen Mycobacterium leprae has the capacity to alter the developmental reprogramming of lineage committed host glial cells to progenitor/stem cell-like cells in mammals (Hess and Rambukkana 2015). In addition, the host–pathogen symbiosis commonly recorded between bacteria and stem cells of the intestine, where microbial products can stimulate stem cell survival, trigger regeneration and provide protection against stress (Nigro et al. 2014), or the ways in which Escherichia coli cells can mobilize functional hematopoietic stem cells (Burberry et al. 2014), are but two of many examples of what may be a widespread but poorly understood phenomenon in animals.

3. Discussion and Future Perspectives

Here, we showed that the intracellular symbionts (either prokaryotic or eukaryotic) of sponges are found only in representative species of Demospongiae, one of the four Porifera classes (Demospongiae, Hexactinellida, Homoscleromorpha and Calcarea; Table 2). Prokaryotic organisms are found in specialized cells—bacteriocytes—in representatives of different orders of Demospongiae (Table 2). Bacteriocyte-like cells have been reported in four phyla, indicative of their independent evolutionary origins (Figure 2). Eukaryotic symbionts are exclusively unicellular photosynthetic algae in sponges, and are found in pluripotent stem cells, most frequently in the archaeocytes; they are documented in five orders: Spongillida, Haplosclerida, Clionaida, Suberitida, and Tetractinellida (Table 1). It is interesting to note that the representatives of the green algae from the phylum Chlorophyta were found only in freshwater sponges of the order Spongillida.

Table 2. Distribution of bacteriocytes harboring intracellular symbiotic bacteria within Porifera.

Order	Species	Bacteriocyte Localization	Stem-Cell	References
		Class Demospongiae		
Biemnida	*Biemna ehrenbergi*	Mesohyl	No	(Ilan and Abelson 1995)
	Neofibularia irata	Mesohyl	No	(Wilkinson 1978)
Axinellida	*Cymbastella concentrica*	Mesohyl	No	(Nguyen et al. 2014)
Verongiida	*Aplysina cavernicola*	Mesohyl	No	(Vacelet 1975)
	Aplysina aerophoba	Mesohyl	No	(Vacelet 1975)
	Aplysina cauliformis	Mesohyl	No	(Gochfeld et al. 2019)
	Aplysina fistularis	Mesohyl	No	(Negandhi et al. 2010)
Haplosclerida	*Petrosia ficiformis*	Mesohyl	No	(Vacelet and Donaday 1977)
	Haliclona tubifera	Larva	No	(Woollacott 1993)
	Haliclona cnidata	Mesohyl	No	(Schellenberg et al. 2020)
	Haliclona sp.	Mesohyl	No	(Tianero et al. 2019)
	Oceanapia sagittaria	Mesohyl	No	(Salomon et al. 2001)
	Cribochalina	Mesohyl	No	(Rützler 1990)
Chondrosida	*Chondrosia reniformis*	Mesohyl	No	(Lévi and Lévi 1976)
Chondrillida	*Halisarca dujardinii*	Mesohyl	No	(Ereskovsky unpublished)
	Halisarca restingaensis	Mesohyl	No	(Alvizu et al. 2013)
	Chondrilla australiensis	Mesohyl and Larva	No	(Usher and Ereskovsky 2004)
Suberitida	*Suberites domuncula*	Mesohyl	No	(Bohm et al. 2001)
Tetractinellida	*Thoosa* sp., *Alectona* sp.	Mesohyl	No	(Garrone 1974)
	Jaspis stellifera	Mesohyl	No	(Wilkinson 1978)
Tethyida	*Tethya stolonifera*	Mesohyl	No	(Taylor et al. 2021)
Poecilosclerida	*Lycopodina hypogea*	Mesohyl	No	(Vacelet and Boury-Esnault 1996)
	Cladorhiza sp.	Mesohyl	No	(Vacelet et al. 1996)
	Crambe crambe	Mesohyl	No	(Maldonado 2007)
	Hymedesmia methanophila	Mesohyl	No	(Rubin-Blum et al. 2019)
Scopalinida	*Svenzea zeae*	Mesohyl and Larva	No	(Rützler et al. 2003)
	Scopalina ruetzleri	Mesohyl	No	(Rützler et al. 2003)
Agelasida	*Astrosclera willeyana*	Mesohyl	No	(Worheide 1998)
Demospongiae incertae sedis	*Myceliospongia araneosa*	Mesohyl	No	(Vacelet and Perez 1998)

It is generally accepted that all multicellular organisms actively coordinate somatic maintenance properties, including growth (in organisms with indeterminate growth -such as sponges, corals, and the immortal *Hydra*- throughout the organism´s life span; (Vogt 2012)); cell proliferation and cell death for tissue homeostasis; and for phenomena such as regeneration, with ASCs in some of these organisms acting as the building blocks for all needs (Biteau et al. 2011; Merrell and Stanger 2016; Rinkevich et al. 2022). The additional cellular homeostasis required for the management and coordination of intracellular symbiosis clearly presents a scenario in which non-traditional functions were imposed on ASC performance during evolution. In contrast to the sponge examples, most other ASC types do not possess intracellular symbionts and yet directly or indirectly influence cytosymbiosis in a wide range of marine and terrestrial taxa (but see *Wolbachia* infections in isopods that harbour these intracellular parasites not only inside haemocytes but also within ASCs of the hematopoietic system, or the *Wolbachia* that highjack the female germline of insects (Chevalier et al. 2011; Ote and Yamamoto 2020). Indeed, intracellular symbionts are rarely associated with ASCs, and to our knowledge—with the exception of sponges—only in the case of bacterial symbionts (Figure 2).

It is, therefore, of great interest to illuminate the mechanisms driving the highly coordinated behaviours of ASCs in specific symbioses, such as the unipotent bacteriocyte stem cells that continuously proliferate to produce new bacteriocytes in some annelids (Bright and Giere 2005); the maintenance of symbiosis during the continuous bacteriocyte formation from aposymbiotic neoblasts in adult paracatenulid flatworms (Dirks et al. 2012); the epithelial stem cells that actively shape the microbial intracellular communities in Hydra (Fraune et al. 2009); or the larval bacteriocytes that develop from uninfected putative stem cells in the rice weevil Sitophilus oryzae (Alvizu et al. 2013). Thus, cytosymbiosis-borne ASC phenomena are either established (in sponges) or supported (directly and indirectly; at least in Cnidaria, Platyhelminthes, Annelida, Arthropoda [insects and crustacean alike], Urochordata and Vertebrata). However, the most prominent examples of endosymbiotic ASCs come from the sponges.

Many challenges remain in studying symbioses at the mechanistic level. First, it should be possible to isolate and culture host and symbiont separately; this is rarely possible. Many symbioses have arisen in inhospitable environments (e.g., deep sea Bathymodiolus mussels and their sulphide- and methane-oxidizing bacterial symbionts, (Duperron et al. 2006), which cannot be easily recreated in the laboratory. Marine algae are particularly difficult to culture, and yet are the basis for many photosymbiotic associations. In addition, many symbioses are obligate, or transmitted vertically, making them near impossible to manipulate without killing host or symbiont, or affecting embryonic survival. It should be possible to generate aposymbiotic and symbiotic hosts at will to understand the metabolic and genetic changes directly caused by symbiosis. Such studies on sponges have recently been initiated (Geraghty et al. 2021; Hall et al. 2021). Finally, from a technical perspective, it is often difficult to separate host and symbiont genomes in intracellular symbioses. In particular, RNA sequencing of endosymbiotic host tissues en masse fails to adequately define transcriptional profiles at the fine resolution necessary to assess changes at the cellular level.

In spite of these many limitations, metagenomic approaches are now giving new insight into host–symbiont interactions. For instance, dual RNA-seq combined with imaging has allowed the time course of endosymbiont-embryonic host cellular interactions to be mapped during cereal weevil metamorphosis (Maire et al. 2020). RNAseq of aposymbiotic and symbiotic bobtail squid tissues as well as Vibrio both before and after venting from the light organ (Thompson et al. 2017) and hybridization chain reaction-fluoresencent in situ hybridization of both partners at the onset of symbiosis maps transcriptional changes in situ (Nikolakakis et al. 2015). A recent RNASeq analysis, combined with electron and confocal microscopy of fresh-water demosponge model Ephydatia muelleri, has revealed some of the genetic pathways involved in intracellular host/photosymbiont interactions, identifying putative genetic pathways involved with endosymbiosis establishment (Hall et al. 2021). RNASeq analysis and comparative analyses of the transcriptomes of aposymbiotic and symbiotic sponges have identified a suite of genes that are regulated at the early establishment stages of the stable symbiosis between E. muelleri and its native green algal symbionts (Geraghty et al. 2021). Authors have also begun to differentiate these genes from those involved in generalized phagocytosis events related to feeding and/or immunity. Single cell analyses are providing new avenues for understanding that might be well suited to tackling the ASC/endosymbiont–poriferan mutualism. As a case in point, recent work on the cnidarian coral Xenia has identified the cell lineage containing the Symbiodinium algal symbiont as originating as a pre-endosymbiotic progenitor pool (Hu et al. 2020). Similarly, Levy et al. (2021) simultaneously queried the transcriptomes of Symbiodinium-containing host cells and their symbionts, and compared with “free" Symbiodinium and non-symbiotic gastrodermal cells and in the stony coral Stylophora pistillata. They identified shared lipid metabolism pathways in algal hosting cells with those of Xenia (Hu et al. 2020) and Exaiptasia pallida (Hambleton et al. 2019), a symbiotic anemone, suggesting cnidarian-dinoflagellate photosymbioses may generate very particular constraints on physiologies despite their independent evolutionary origins. Similar efforts in sponges would thus add considerable insight into the establishment and maintenance of photosymbioses, and provide new insight into the diversity of symbiotic associations seen across the tree of life.

Author Contributions: A.E., I.M.L.S. and B.R. conceived the work, wrote the manuscript and approved the final version. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the European Cooperation in Science & Technology program (EU COST). Grant title: “Stem cells of marine/aquatic invertebrates: from basic research to innovative applications” (MARISTEM). The work of AE was conducted under the IDB RAS Government basic research program in 2021 No. 0088-2021-0009.

Conflicts of Interest: The authors declare no conflict of interest.

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