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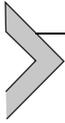
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From traveler to homebody: Which signaling mechanisms sponge larvae use to become adult sponges?

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Abstract

Cell-to-cell signaling is responsible for regulation of many developmental processes such as proliferation, cell migration, survival, cell fate specification and axis patterning. In this article we discussed the role of signaling in the metamorphosis of sponges with a focus on epithelial–mesenchymal transition (EMT) accompanying this event. Sponges (Porifera) are an ancient lineage of morphologically simple animals occupying a basal position on the tree of life. The study of these animals is necessary for understanding the origin of multicellularity and the evolution of developmental processes. Development of sponges is quite diverse. It finishes with the metamorphosis of a free-swimming larva into a young settled sponge. The outer surface of sponge larvae consists of a ciliated epithelial sheath, which ensures locomotion, while their internal structure varies from genus to genus. The fate of larval ciliated cells is the

most intriguing aspect of metamorphosis. In this review we discuss the fate of larval ciliated cells, the processes going on in cells during metamorphosis at the molecular level and the regulation of this process. The review is based on information about several sponge species with a focus on *Halisarca dujardini*, *Sycon ciliatum* and *Amphimedon queenslandica*. In our model sponge, *H. dujardini*, ciliated cells leave the larval epithelium during metamorphosis and migrate to the internal cell mass as amoeboid cells to be differentiated into choanocytes of the juvenile sponge. Ciliated cells undergo EMT and internalize within minutes. As EMT involves the disappearance of adherens junctions and as cadherin, the main adherens junction protein, was identified in the transcriptome of several sponges, we suppose that EMT is regulated through cadherin-containing adherens junctions between ciliated cells. We failed to identify the master genes of EMT in the *H. dujardini* transcriptome, possibly because transcription was absent in the sequenced stages. They may be revealed by a search in the genome. The master genes themselves are controlled by various signaling pathways. Sponges have all the six signaling pathways conserved in Metazoa: Wnt, TGF-beta, Hedgehog, Notch, FGF and NO-dependent pathways. Summarizing the new data about intercellular communication in sponges, we can put forward two main questions regarding metamorphosis: (1) Which of the signaling pathways and in what hierarchical order are involved in metamorphosis? (2) How is the organization of a young sponge related to that of the larva or, in other words, is there a heredity of axes between the larva and the adult sponge?

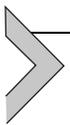
1. INTRODUCTION

Sponges (Porifera), which were among the first multicellular animals, are a sister group to all other Metazoa (Feuda et al., 2017). They are an ancient phyletic lineage of morphologically simple animals that diverged from other metazoans at least 700 Mya, well before the Cambrian explosion (Simion et al., 2017). Sponges occupy a key phylogenetic position, and the study of their molecular organization is a powerful tool for elucidating the first steps of the evolution of multicellularity. There is direct evidence of the origin of Metazoans from protists, and the first multicellular animals are sponges (Sebé-Pedrós, Degnan, & Ruiz-Trillo, 2017).

Although sponges are quite unlike other metazoans anatomically, their embryonic and larval development is surprisingly similar to that of higher animals. For instance, sponges express virtually all the morphogenetic movements observed during embryonic development of Eumetazoa (Ereskovsky, Renard, & Borchellini, 2013). Yet there are relatively few omics data on sponges, and it is difficult to perform a meaningful comparative analysis with bilaterians.

The phylum Porifera is divided into four classes. The Calcarea are sponges with a calcareous skeleton. The Hexactinellida are syncytial glass sponges, whose skeleton consists of silicone dioxide. The Demospongiae are “common” sponges, and the Homoscleromorpha comprises sponge species possessing a basement membrane and metazoan-like cell junctions. Morphogenesis during embryonic development is highly diverse in different orders. Larvae, the only motile stage in the life cycle of sponges, are as diverse as the developmental types, with nine larval morphotypes being distinguished (Ereskovsky, 2010).

Our model organism, *Halisarca dujardini* Johnston, 1842 (Demospongiae) is a small sponge encrusting stones or algae in the sublittoral of boreal and Arctic seas (Fig. 1A). The embryonic development and metamorphosis of *H. dujardini* is well documented (Chen, 1976; Ereskovsky & Gonobobleva, 2000; Lévi, 1956; Metschnikoff, 1879; Schulze, 1877) (Fig. 1B and C). Transcriptomes of adults and larvae as well as a draft of genome sequenced with Illumina technology are available (Borisenko, Adamski, Ereskovsky, & Adamska, 2016). Although metamorphosis of numerous sponges was described at morphological level, the only study of this process with cell-type specific protein markers was performed on *H. dujardini* (Mukhina, Kumeiko, Podgornaya, & Efremova, 2006). Keeping in mind all information available on *H. dujardini* including data on morphology, transcriptome, and protein markers of larval cell types, we tried to analyze and compare the known facts about molecular mechanisms of sponge larva metamorphosis. In this review we focused on the signaling mechanisms involved in metamorphosis since the major morphogenetic events such as changes of the cell shape, rearrangement and migration of cells and changes in the gene expression profiles should be related to cell-to-cell communication.



2. EMBRYONIC DEVELOPMENT

Three model species of sponges were considered in the review: *Halisarca dujardini*, *Sycon ciliatum* and *Amphimedon queenslandica*. Developmental mode is remarkably different in each of these species.

H. dujardini is a viviparous species. Embryos develop inside temporary embryonic capsules formed from dedifferentiated choanocytes (Korotkova & Apalkova, 1975) (Fig. 1B and C). Cleavage is complete, equal and polyaxial (Fig. 1C(b–c)) (Ereskovsky, 2010). A single-layered

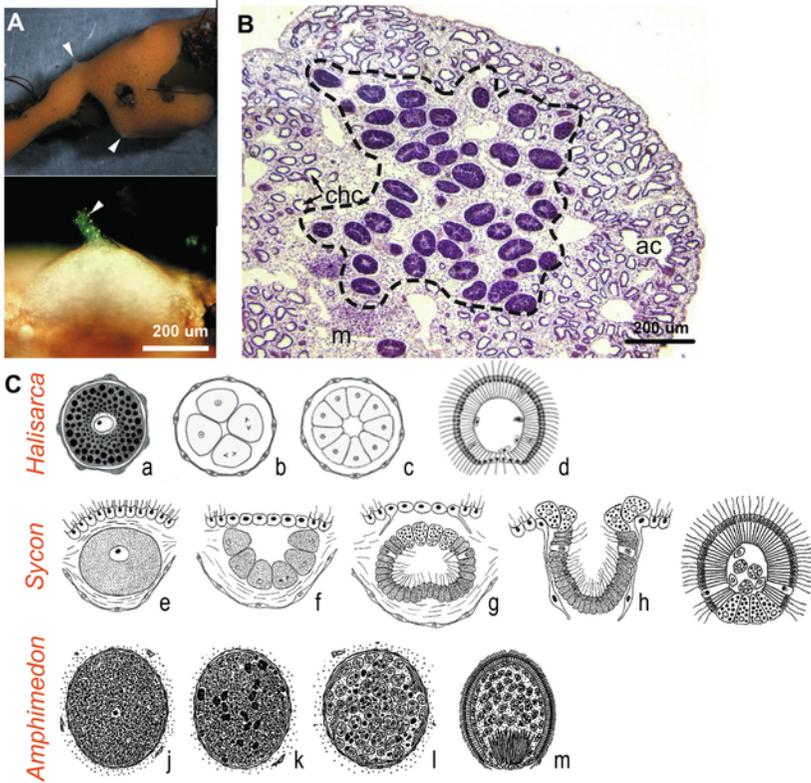


Fig. 1. (A and B). Sponge *Halisarca dujardini* from the White Sea. (A) *Top*—adult sponge on brown algae *Fucus vesiculosus*. Sponges have a canal system, through which they pump water. Water enters the sponge through pores, flows along the canals to choanocyte chambers and exits through large openings (oscula). Oscula are marked with arrowheads. *Bottom*—Rhagon, a young sponge just after metamorphosis. It has only a few choanocyte chambers and one osculum. (B) A sponge with developing larvae (a group of larvae is outlined with a dotted line). The aquiferous system in the central and the basal part of the sponge is destroyed, and normal tissue organization remains only at the periphery. ac, aquiferous canal; chc, choanocyte chambers; m, mesohyl. (C) Developmental stages of sponges with described developmental signalings from classes Demospongiae (*Halisarca* and *Amphimedon*) and Calcarea (*Sycon*; a scheme). a, e, j: egg; b, c: polyaxial cleavage; d: coeloblastula-like larva; f: incurvational cleavage; g, h: stomoblastula and its excurvation; j: amphiblastula larva; k: unequal and asynchronous cleavage; l: layers formation by delamination in morula; m: parenchymella larva. *Panel (C): Modified from Ereskovsky, A. V. (2010). The Comparative Embryology of Sponges. Dordrecht: Springer.*

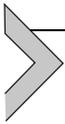
coeloblastula with a small cavity is formed, and outer cells become columnar in shape and ciliated.

The larvae start to move a few days before leaving the parent sponge. The walls of the embryonic capsule soon fuse with those of the aquiferous canal, and larvae escape through the osculum. Released larvae continue to swim freely by moving forward and rotating clockwise around the antero-posterior axis. The larva of *H. dujardini* is rounded and measures about 120–130 μm in diameter. It is evenly ciliated, except at the posterior pole, which is ciliated less densely (Fig. 3B). Its external cells are connected with belt desmosome-like junctions (zonula adhaerens) in their apical parts (Gonobobleva, 2007; Gonobobleva & Ereskovsky, 2004a, 2004b).

The larvae of *S. ciliatum* develop in the mesohyl between the choanocyte and the pinacocyte layer (Fig. 1C(e–h); Franzen, 1988; Leys & Eerkes-Medrano, 2005). Early cleavage is equal and highly stereotypic. The first four blastomeres form a rhomboid arranged in the plane defined by the choanocyte and the pinacocyte layers. Since the third cleavage the mitotic spindle turns to divide daughter cells laterally and in the direction of choanoderm (Fig. 1C(f–g)). This results in the formation of a cup-shaped stomoblastula embryo with an opening directed to the choanoderm. By the end of cleavage and differentiation, the embryo is composed of three cell types: large, granular, non-ciliated macromeres adjacent to the choanocytes; smaller and more numerous micromeres, which have cilia pointing into the embryonic cavity; and four cruciform cells. The embryo then undergoes excurvation (inversion) through stomoblastula opening (Fig. 1C(h)). As a result of this process, during which the embryo is turned inside out, the cilia are positioned on the outer larval surface and the larva is moved to the choanocyte chamber. During metamorphosis, the larva settles on the anterior pole; within minutes the ciliated cells of the anterior half undergo epithelial–mesenchymal transition (EMT) and form the inner cell mass. In contrast, the macromeres maintain their epithelial organization, completely enclose the micromeres, and become the pinacocytes of the forming juvenile.

The embryos of *A. queenslandica* also develop in brooding chambers in follicular capsules (Fig. 1C(j); Adamska et al., 2007; Leys & Degnan, 2002). At the early stages of cleavage, chaotically arranged micromeres and macromeres of different size are formed (Fig. 1C(k)). By the end of cleavage, the morula (solid blastula) already contains micro- and

macromeres, amoeboid cells, sclerocytes, ciliated cells and pigment cells (Fig. 1C(l)). All these cells are mixed until positional signals guide them to their definitive place in the larvae. Cleavage is followed by the formation of cell layers due to differential cell movements in the embryo. The main results of these movements are the formation of an outer ciliated cell layer by ciliated cells and an accumulation of pigment cells and spicules, referred to as the pigment spot, at the posterior pole of the embryo (Fig. 1C(m)). The outer ciliated cells will provide locomotion of the larva, while the pigment spot will transform into a pigment ring, a circular arrangement of pigmented cells with a photosensory function. By this stage the embryo elongates along the antero-posterior axis, the pigment ring marking the posterior pole. Many of the developmental genes expressed in the embryo are closely related to the pigment ring.



3. MORPHOLOGY OF THE METAMORPHOSIS

Metamorphosis is a process in the postembryonic period during which the larva develops into a juvenile. Fundamental morphological and physiological changes occurring during metamorphosis involve a reorganization of the basic body plan.

The most intriguing aspect of metamorphosis is the mechanism of morphogenetic movement, which results in the formation of the main definitive structures. Delage proposed the hypothesis of the “inversion of the germ layers” in sponges late in the 19th century (Delage, 1892). He described an inward migration of superficial ciliated cells during metamorphosis in some Demospongiae larvae and suggested that these cells formed the choanoderm. “Inversion” meant that external larval cells formed internal gut-like structures of an adult sponge. Delage's observations have been confirmed and disproved several times. The fate of ciliated cells and the formation of definitive tissues in sponges is still debated (Efremova, 1997; Ereskovsky, 2010; Ereskovsky & Dondua, 2006; Leys, 2004; Leys & Ereskovsky, 2006; Sogabe, Nakanishi, & Degnan, 2016). However, it has been demonstrated that the fate of ciliated cells depends on the larval organization (reviewed in Ereskovsky, 2010). This means that no single interpretation of their fate during metamorphosis can be proposed.

The larvae of *H. dujardini* attach to the substrate with their anterior pole and flatten along the antero-posterior axis. The ciliated cells of the

posterior pole form the exopinacoderm (Fig. 2A–C). Those of the anterior hemisphere flatten parallel to the substrate (Fig. 2B and C). An inner conglomerate, a mass of ciliated cells of the anterior pole, the internal cells and the ciliated cells of the internal larval cavity, is formed below the exopinacoderm (Fig. 2B). By the end of metamorphosis, the cells of this conglomerate differentiate into choanocytes, endopinacocytes, basopinacocytes and mesohyl cells (Gonobobleva & Ereskovsky, 2004a). Calcium signaling plays an important role in initiation of metamorphosis in another demosponge, *Amphimedon queenslandica* (Nakanishi, Stoupin, Degnan, & Degnan, 2015).

Cytological markers have been successfully used to describe the differentiation of some cells of sponge larvae (Amano & Hori, 1993, 1996, 2001; Ereskovsky, Konjukov, & Willenz, 2007; Ereskovsky, Tokina, Bézac, & Boury-Esnault, 2007; Gaino, Baldaconi, & Corriero, 2007; Usher & Ereskovsky, 2005). Vital fluorescent non-specific tracer CMFDA was used to label larval peripheral cells (Leys & Degnan, 2002). Elegant experiments combining EdU-labeling of newly synthesized DNA with lipophilic tracer CM-Dil were performed on *A. queenslandica*, the first demosponge whose genome was sequenced (Sogabe et al., 2016). The authors followed the fate of ciliated cells to adult choanocytes and showed that they transformed into mesenchymal pluripotent stem cells resembling archaeocytes. Some of these EdU-labeled cells differentiated in choanocytes, but other, unlabeled cells were also involved in the formation of choanocyte chambers. These results indicate that multiple lineages of larval cells usually contribute to the formation of individual choanocyte chambers during metamorphosis, con-

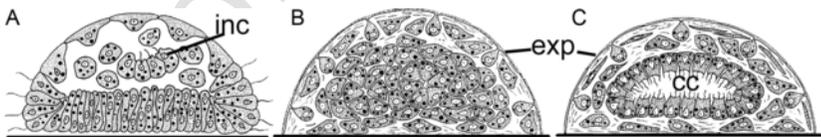


Fig. 2. Schematic drawing of the main stages of metamorphosis in *Halisarca dujardini*. (A) First stage, immediately after the attachment of the disphaerula and the destruction of the internal cavity (inc); cells of larval posterior pole (pci) begin to flatten to form exopinacoderm. (B) Pupa with developing exopinacoderm. (C) Early rhagon, which has a single choanocyte chamber (cc) and the exopinacoderm (exp), but lacks the basopinacoderm. From Gonobobleva, E., & Ereskovsky, A. (2004a). *Metamorphosis of the larva of Halisarca dujardini (Demospongiae, Halisarcida)*. *Bulletin de l'Institut Royal Des Sciences Naturelles de Belgique—Bulletin van Het Koninklijk Belgisch Instituut Voor Natuurwetenschappen*, 74, 101–115, reproduced with permission of l'Institut Royal des Sciences Naturelles de Belgique.

tradicting previous reports that in other sponge species choanocyte chambers form clonally (Nakanishi et al., 2015).

Molecular markers are now available for sclerocytes (cells producing the mineral skeleton) of *Ephydatia fluviatilis* and *Sycon ciliatum* (Funayama, Nakatsukasa, Kuraku, et al., 2005; Voigt et al., 2017) and archaeocytes of *E. fluviatilis* (Funayama, 2010). Annexin was shown to be a specific choanocyte marker, and archaeocytes were found to be the precursors of choanocytes during asexual reproduction of the freshwater sponge *E. fluviatilis* (Funayama, Nakatsukasa, Hayashi, & Agata, 2005). However, few of these molecules seem to be expressed in a lineage-restricted manner: homologs of *piwi* and *annexin* are present in *H. dujardini*, but they are not specifically expressed in, respectively, archaeocytes and choanocytes (GenBank accession numbers MH006695 for *piwiA*, MH107849 for *piwiB* and MH107850 for *annexin*). In general, the range of the available markers is very limited. This means that sponge cell lineages, especially those involved in development and reproduction, are poorly understood.



4. THE FATE OF CILIATED CELLS DURING METAMORPHOSIS OF *H. DUJARDINI*

The metamorphosis of *H. dujardini* was described using light and electron microscopy (Ereskovsky, Konjukov, & Willenz, 2007; Ereskovsky, Tokina, et al., 2007; Gonobobleva & Ereskovsky, 2004a, 2004b; Lévi, 1956). However, given considerable changes in the morphology of ciliated cells during metamorphosis, molecular markers are obviously required to trace their fate precisely. Intercellular junctions in sponges are calcium-dependent, and sponge tissues dissociate to cells in calcium-free media. A suspension of isolated sponge cells can be easily obtained by placing tissues in artificial seawater without Ca^{2+} supplied with a chelator such as EDTA (Ereskovsky, Konyukov, & Tokina, 2010; Ereskovsky, Konjukov, & Willenz, 2007; Ereskovsky, Tokina, et al., 2007; Mukhina et al., 2006; Mukhina, Kumeiko, Podgornaya, & Efremova, 2007). The larvae of *H. dujardini* were dissociated, and the cell suspension was overlaid onto discontinuous density gradient made with Percoll solution and centrifuged. The gradient consisted of layers from 10% to 45% Percoll in calcium-free seawater, producing five fractions numbered from top to bottom of a tube (Fig. 3).

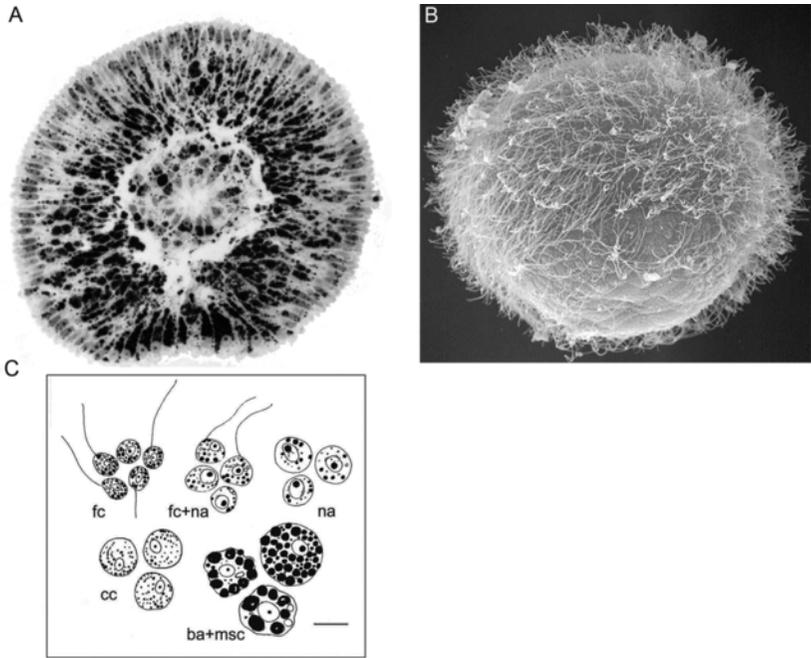


Fig. 3. Larvae of *H. dujardini*, their cell composition and the ability of cells to aggregate. (A) Semi-thin longitudinal section of the free-swimming disphaerula larva. ba: blastomere-like amoebocytes; ci: cilia; cil: ciliated cells; ich: internal ciliated cells cavity, msc: maternal spherulous cells; na: nucleolated amoebocytes; pci: posterior flagellated cells; ap, anterior pole; pp, posterior pole. Scale bar: 20 μ m. (B) SEM of disphaerula larva. ci, cilia; pci, posterior flagellated cells; ap, anterior pole; pp, posterior pole. Scale bar: 20 μ m. (C) Scheme of larval cell fractions of *H. dujardini* obtained by Percoll density gradient centrifugation. Scale bar: 5 μ m.

The separation of larval cell types (Fig. 3C) made it possible to determine the polypeptide composition of the fractions by SDS-PAGE. The protein composition of fraction I (ciliated cells) differed dramatically from the rest. There were four major bands in the range of 42–68 kDa. One lane with a molecular weight of 42 kDa probably corresponded to actin. Another double lane of \sim 55 kDa corresponded to α and β tubulin isoforms. A third band of unknown nature with an apparent molecular weight (M_r) of 68 kDa (antigen S, p68) was chosen for polyclonal antibody production. Anti-p68 (S antigen) antibodies produced in rabbit were shown to be specific to larval ciliated cells, and were used in double immunofluorescence together with commercial anti-tubulin antibodies to trace the fate of ciliated cells during metamorphosis.

Double staining of larvae with both antibodies showed that protein p68 (S) was present in granules in the body of ciliated cells but was absent in the cilium. Tubulin was present in the cilium, the basal body and, to a lesser extent, in the cortical cytoskeleton structures (Fig. 4A and D(a)). Thus, anti-S and anti-tubulin antibodies stained different compartments in ciliated cells.

Cilia visualized with anti-tubulin antibody were seen all over the larval surface except at the posterior pole, which was bare and lacked any specific fluorescence (Fig. 4A(a)). Such larvae settled on the substrate and flattened rapidly. In the narrow transparent monolayer of lateral ciliated cells attached to the substrate, the cilia appeared to be submerged into the cytoplasm, *i.e.*, were internalized (Fig. 4A(b–c)). Both anti-S and anti-T staining were confined to the internal cell mass at the middle sections of metamorphosing larvae (Fig. 4B). By 3 days post-settlement most of the ciliated cells joined the newly formed choanocyte chambers (Fig. 4C). S-specific labeling was seen in choanocytes but not in pinacocytes or amoebocytes.

At the morphological level choanocytes differ from ciliated cells by a prominent collar around cilia. S antigen (p68) was concentrated in the apical part of the choanocyte and visualized as rings or curves outlining the apical border (Fig. 4D(c)). Hence, ciliated cells from the anterio-lateral surface of the larva internalized during metamorphosis, and then the cells of the internal cell mass with the same immunoreactivity (S/tubulin-positive) participated in the formation of choanocyte chambers in the juvenile.

Some of the larval ciliated cells appear to be the source of internal cells in juvenile *H. dujardini*. A very unusual polypeptide composition of the ciliated cells, with few prominent major proteins, could be a sign of their terminal differentiation, but no evidence of exfoliation of ciliated cells or their phagocytosis by any other cells during the short time of metamorphosis has yet been found (Gonobobleva & Ereskovsky, 2004b). Alternatively, a unique protein composition may be associated with the preparation of ciliated cells for a precipitous change of their fate. The S protein may be meant for choanocytes, which later become able to synthesize their own protein. The localization of p68 (S) must be checked by electron microscopy and its identity, by mass-spectrometry or Edman degradation procedure. The transcriptome of the adult sponge is available (Borisenko et al., 2016), and since the adult sponge can produce S anti-

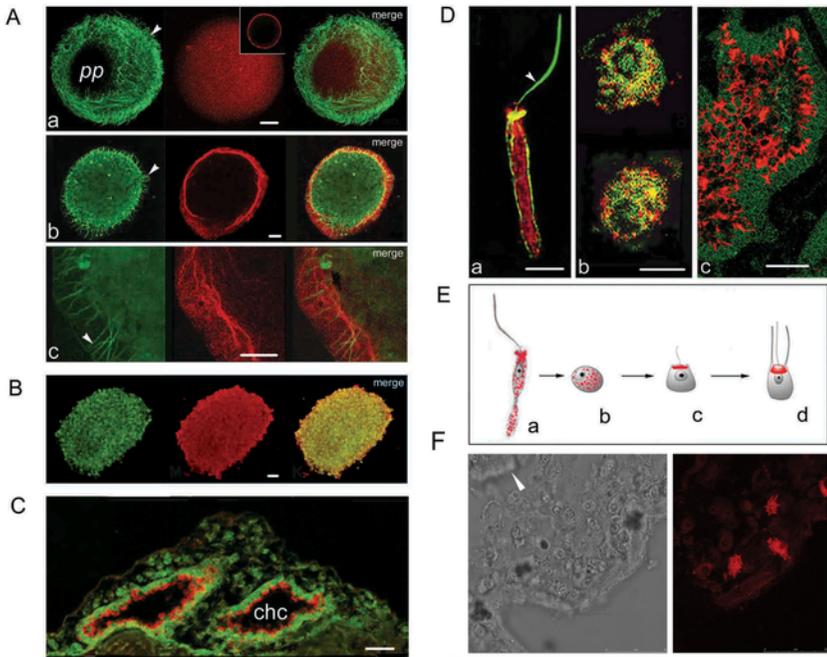
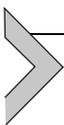


Fig. 4. Free-swimming and metamorphosing larva of *H. dujardini* double-stained with anti-tubulin and anti-p68 antibodies. Sequential steps of larval settlement (A), metamorphosis (B and C), transformation of ciliated cell (D and E) and adult sponge (F). (A) Larval settlement. Laser confocal scanning microscopy of whole mount larvae. a: Free-swimming larvae before settlement; top optical section and midsection (inset). Here and elsewhere green staining indicates tubulin, and red staining indicates S antigen. Arrow points to cilia. b: Larvae 40 min after contact with substrate (glass). c: Peripheral part of flattened larvae. Colors and designations are the same as above. Internalized cilia on c are indicated by arrow. (B) Larvae 6 h after contact with substrate. Ciliated cells are submerged into the larva. Scale bar for all images: 20 μm . (C) Young sponge 3 days after settlement, immunostained paraffin section. chc—choanocyte chamber formed *de novo*. Note the absence of S antigen staining in young pinacoderm. Scale bar 10 μm . (D) Transformation of ciliated cell (a) by inward migration (b) into the choanocyte chamber (c) of a definitive sponge. Scale bar 2 μm for a, b and 20 μm for c. (E) Scheme based on original image (D). a: Ciliated larval cells, b: ciliated cells inside larva, c: choanocyte of young sponge, d: choanocyte of definitive sponge. (F) Adult sponge with non-motile larva (correspond to Fig. 2B). a: DIC microscopy; b: immunofluorescence with anti-S. Pinacocytes are stained but note the absence of staining in cells of the larva before the release (white arrow). Scale bar: 20 μm .

gen (Fig. 4(F)), we may be fairly sure that the S transcript can be found. Once this has been done, we may be able to trace specific cell-type transcription. By now we have proved its specificity for ciliated cells and juvenile choanocytes at the protein level.

Transdifferentiation of cells is common in sponges. For instance, it occurs during regeneration and the formation of primmorphs (Borisenko, Adamska, Tokina, & Ereskovsky, 2015; Ereskovsky et al., 2015; Lavrov, Bolshakov, Tokina, & Ereskovsky, 2018; Lavrov & Kosevich, 2016). Light and electron microscopic data demonstrate that choanoblasts can develop in parenchymella from ciliated cells and archaeocytes in some genera (*Mycale*, *Hamigera*) (Borojevič & Lévi, 1965; Boury-Esnault, 1976), only from archaeocytes in some other taxa (*Baikalospongia*, *Microciona*) (Efremova & Efremov, 1979; Kaltenbach, Kuhns, Simpson, & Burger, 1999), or from an internal population of archaeocytes and larval choanocytes (Spongillidae) (Weissenfels, 1989). A recent study of another demosponge, *A. queenslandica*, has shown that multiple larval cell types contribute to choanocyte chambers of the juvenile (Sogabe et al., 2016). So, while the fate of ciliated cells has been elucidated in *H. dujardini*, it has to be studied in other species with the use of a board range of markers.

Our approach could be extended to the search for new protein markers with the separation of adult cell types and powerful techniques such as 2D-DIGE. Different cell types of the adult sponge clearly possessed specific proteins (Fig. 5, arrows). Proteins in these spots could be identified after excision by mass-spectrometry and compared with the assembled transcriptome of the adult sponge. Then the specific synthesis could be traced by the whole-mount RNA–RNA *in situ* hybridization (Borisenko et al., 2016).



5. A JOURNEY FROM THE OUTSIDE INWARD: HOW CILIATED CELLS BECOME DETACHED

Antero-lateral ciliated cells leave the epithelium of the larva and migrate to the internal cell mass as amoeboid cells to be differentiated into choanocytes and/or other cell types of the juvenile sponge. Intercellular junctions have been found between larval ciliated cells, choanocytes and pinacocytes (Amano & Hori, 1996; Bergquist & Green, 1977; Boury-Esnault, Ereskovsky, Bézac, & Tokina, 2005; Evans, 1977; Ereskovsky et al., 2009; Ereskovsky & Tokina, 2004; Gonobobleva & Ereskovsky, 2004b; Gonobobleva & Maldonado, 2009). In our opinion, this indicates the presence of epithelial–mesenchymal transitions (EMT):

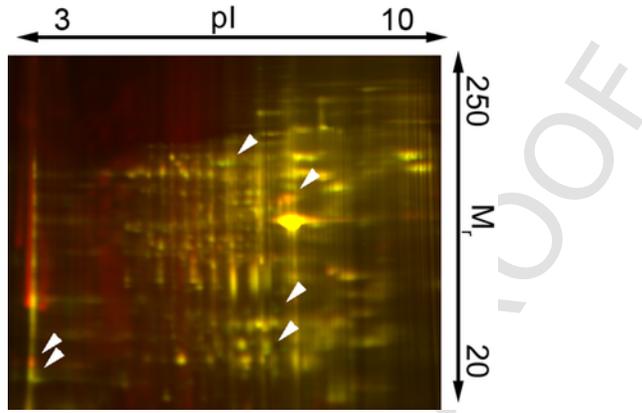


Fig. 5. Difference gel electrophoresis (DIGE; according Alban et al., 2003) revealed cell-specific protein markers. Cell suspension of an adult sponge was separated by centrifugation in density gradient, and proteins were extracted from choanocyte- and archaeocyte-enriched fractions with urea-containing solution. Each protein mixture was covalently labeled with reactive cyanine dye: choanocyte proteins were labeled with Cy3 (green), and archaeocyte proteins, with Cy5 (red). Then protein mixtures were pooled and underwent 2D electrophoresis by isoelectrofocusing and SDS-PAGE. After scanning the gel with lasers of different wavelength, we could recognize proteins specific for choanocytes (green), for amoebocytes (red), or common proteins (yellow).

ciliated cells break free from junctions and leave the polarized sheath to become amoeboid mesenchymal-like cells of the internal mass.

The loss of E-cadherin-mediated adherens junctions is the key event of EMT (Huang, Guilford, & Thiery, 2012). There are numerous proteins with cadherin repeats in their extracellular parts, but unlike true cadherins they have neither delta- nor beta-catenin binding domains in the cytoplasmic part. Proteins containing cadherin repeats have long been known, but the presence of true epithelial cadherin remained a mystery. The presence of E-cadherin has been shown for sponges from all classes (Belahbib et al., 2018). We identified one cadherin homolog in *H. dujardini* transcriptome, which contained multiple cadherin repeats, a transmembrane helix and a cytoplasmic tail with domains for binding intercellular components of junction, delta-catenin (p120 protein) and beta-catenin. The sequence also contains seven EGF-like domains and two laminin domains, which is characteristic of invertebrate cadherin (Fig. 6). So, *H. dujardini*, similarly to other sponges (Belahbib et al., 2017), also has true cadherin, which is the main component of adherens junctions serving as a cell-to-cell lock in epithelial sheath.

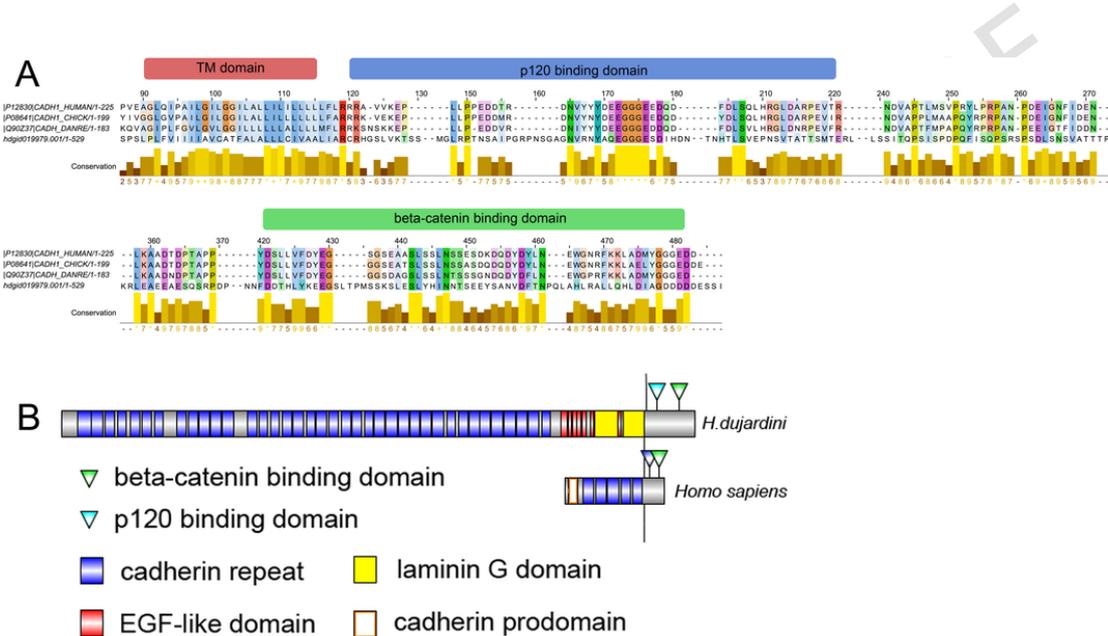


Fig. 6. Comparison of E-cadherin structure in sponge *H. dujardini* and bilateria. (A) Alignment of the cytoplasmic parts of E-cadherins in humans, chick, zebrafish and *H. dujardini*. Conserved domains responsible for interaction with delta-catenin (p120) and beta-catenin, present in the intracellular part of E-cadherin molecule in *H. dujardini*, anchor adherens junctions to the cytoskeleton. (B) Domain organization of E-cadherins in humans and sponges. Invertebrates cadherins are characterized by multiplication of conservative cadherin repeats and the presence of laminin G-like domains, while lacking the prodomain, a short N-terminal sequence releasing with proteolytic maturation. Sequence from *H. dujardini* was identified in transcriptome with local BLAST (option tblastn) and domains identified with SMART and InterPro 70.0 (EMBL-EBI); GenBank accession number is MH107848.

The repression of E-cadherin expression is followed by the disappearance of adherens junctions after the start of EMT (Huang et al., 2012). EMT is controlled by the master genes *snai1/snai2*, *twist* and *ZEB* (Lamouille, Xu, & Derynck, 2014). Their expression induces EMT in many processes associated with development, regeneration and diseases. Snai and ZEB are zinc finger transcription factors. Snai has a SNAG domain and sites for binding LATS2, KPNB1, NOTCH1 and PARP1 proteins; ZEB possesses an atypical homeobox and/or Glu-rich C-end. There are many transcripts for zinc finger DNA-binding proteins in *H. dujardini* transcriptome but none of them has the characters typical of Snai and ZEB. A similar situation observed for Twist. It is a basic helix-loop-helix (bHLH) transcription factor (TF), and *H. dujardini* has a lot of bHLH TFs, but they have only the DNA-binding domain in common with Twist. We failed to identify the master genes of EMT in *H. dujardini* transcriptome, possibly because transcription was absent during the sequenced staged. They may be revealed by a search in the genome.

Master genes themselves are controlled by various signaling pathways, mainly TGF-beta, FGF and Wnt. Finally, the process of internalization of flagellated cells during metamorphosis is under control of intercellular signals.



6. SIGNALING PATHWAYS IN SPONGES

Cell-to-cell communications regulate many processes during development and adulthood of metazoans. During embryogenesis intercellular signals are the third main informational input to developmental process after maternal factors and zygotic genome. Key processes such as proliferation, cell migration and survival, cell fate specification and axis patterning are managed by cell-to-cell signaling. These processes are also regulated through intercellular communication. Six signaling pathways are conserved across Metazoa: Wnt, TGF-beta, Hedgehog, Notch, FGF and NO-dependent. Here we summarize facts and assumptions about each of these pathways in sponges.

Wnt signaling is the best studied pathway in sponges due its probable role in axial patterning, especially in early branching Metazoa. Representatives of all the four poriferan classes are studied for Wnt ligands. Syncytial sponges, Hexactinellida, have no Wnt ligands although they have all the other components of this pathway (Schenkelaars et al., 2017;

Windsor Reid et al., 2018). Distribution of ligands and receptors is quite different even between genera. For instance, *A. queenslandica* (Demospongiae) has two Wnt ligands, three Frizzled receptors, a co-receptor LRP5/6, a secondary messenger Disheveled, beta-catenin, cytoplasmic beta-catenin destruction complex (GSK3, APC, Axin), nuclear factors TCF and Groucho (Adamska et al., 2010). Freshwater sponge *Ephydatia muelleri* has three ligands and three Frizzled receptors (Windsor Reid et al., 2018). In our model demosponge, *H. dujardini*, there are ten ligands and five receptors (Borisenko et al., 2015). Cytoplasmic scaffold proteins Axin and APC have similar features in both species. The beta-catenin binding site not found in Axins, and most of the conserved domains except armadillo repeats are not found in APCs. Biochemical studies on poriferan beta-catenin demonstrate that intermolecular interactions during cytoplasmic signal transduction can be mediated by highly diverged sequences (Nichols, Dirks, Pearse, & King, 2006; Schippers & Nichols, 2018). This may be a reason why such domains cannot be recognized by ordinary bioinformatic methods.

The champion in Wnts complexity, a calcisponge *Sycon ciliatum*, contains 21 ligands. In addition to ligands, four receptors and all core intracellular components except Axin are described in *S. ciliatum* (Leininger et al., 2014). Sponges from the genus *Oscarella* (Homoscleromorpha) have from 3–8 ligands (Lapébie et al., 2009; Leininger et al., 2014). The presence of other pathway components has not been ascertained.

Phylogenetic analysis cannot place poriferan Wnt in any of the established subfamilies of metazoan ligands (Adamska et al., 2010; Lapébie et al., 2009; Leininger et al., 2014). Phylogeny inference made with sponge sequences only demonstrates the presence of Porifera-specific subfamilies (Windsor Reid et al., 2018). In demosponges Wnts form three subfamilies (named PorWntA-C), in homoscleromorphs, two families (WntI-II), an in calcisponges, 21 families (CWntA-U). Notably, the nine of the ten *H. dujardini* ligands cannot be classified within any of these families.

Wnt ligands are expressed in embryos, larvae and adult sponges. The expression of some Wnt ligands was shown in adult and larvae of *H. dujardini* (Fig. 7A and B). Two ligands, *HdWntD* and *HdWntE*, were expressed in the oscular tube, while *HdWntG* transcripts were observed in exopinacocytes. In addition, a set of *Wnt* is expressed in the posterior

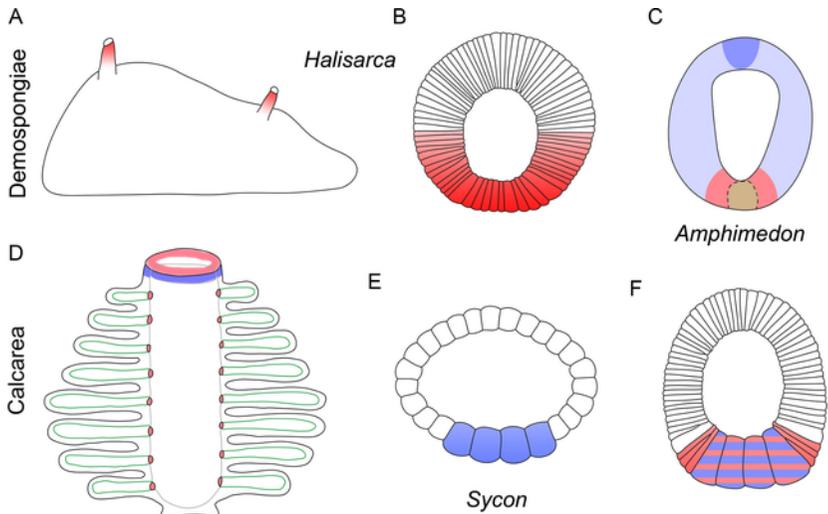


Fig. 7. Summarized data about axial-related expression of *wnt* and *TGF-beta* in three model sponges from Demospongiae (A–C) and Calcarea (D and F) classes. *Wnt* ligands are red and *TGF-beta* is blue; brightness depends on expression intensity. For embryo and larvae anterior end is oriented at top and posterior at bottom. (A and B) Main expression patterns of *HdWnt* is in oscular tube at adult *H. dujardini*, and posterior half of larva. Some *wnt*'s are expressed in basal part of sponge, and in exopinacoderm in “salt and pepper” pattern as well (data not shown). Expression patterns of *TGF-beta* are unknown in *Halisarca* yet. (C) expression of *AqWnt* and *AqTGFb* in *A. queenslandica* embryo at the pigment spot formation stage. (D) Adult *Sycon ciliatum* sponge demonstrate oscular expression of *wnt* and *TGF-beta* ligands; two *wnt*'s expresses in proximal part of choanocyte chambers as well. (E) Only *TGF-beta* transcript presented at stomoblastula stage, in aboral macromeres; *wnt* expression is not detected. (F) expression of both ligands at posterior pole of larva, with broader pattern of *wnt*. Panels (A) and (B): Modified from Borisenko, I., Adamski, M., Ereskovsky, A., & Adamska, M. (2016). Surprisingly rich repertoire of *Wnt* genes in the demosponge *Halisarca dujardini*. *BMC Evolutionary Biology*, 16, 123; Kozin, V. V., Borisenko, I. E., & Kostyuchenko, R. P. (2019). Establishment of the Axial Polarity and Cell Fate in Metazoa via Canonical *Wnt* Signaling: New Insights from Sponges and Annelids. *Biological Bulletin*, 46(1), 12; Panel (C): Modified from Adamska, M., Degnan, S. M., Green, K. M., Adamski, M., Craigie, A., Larroux, C., & Degnan, B. M. (2007). *Wnt* and *TGF-β* Expression in the Sponge *Amphimedon queenslandica* and the Origin of Metazoan Embryonic Patterning. *Plos One*, 2(10), e1031; Panel (D)–(F): Modified from Leininger, S., Adamski, M., Bergum, B., Guder, C., Liu, J., Laplante, M., et al. (2014). Developmental gene expression provides clues to relationships between sponge and eumetazoan body plans. *Nature Communications*, 5. <https://doi.org/10.1038/ncomms4905>.

hemisphere of the larvae: *HdWntL* at the posterior pole, *HdWntK* from the equator to the latest lateral flagellated cells, and *HdWntJ* in a belt-like pattern at the equator (Borisenko et al., 2015). In *A. queenslandica*

AqWntA is first expressed in micromeres throughout the embryo in a diffuse manner. During the development these cells migrate into the inner part of the embryo, then to the outer cell layer, and eventually turn up at the further posterior pole of the larva (Fig. 7C; Adamska et al., 2007, 2010).

Unlike *Amphimedon*, in *S. ciliatum* most *Wnt* expressed in oocytes and early cleavage stages, though transcripts disappeared by the end of embryonic development and before larvae formation. Expression domains of three *Wnt* are associated with the posterior half of larvae (Fig. 7E–F). Two out of five *Frizzled* receptors expressed in micromeres, at the anterior pole of the embryo. Such combinatorial expression of different ligands and receptors allow diversifying results from use of limited set of signaling molecules. Another set of *Wnt* is expressed in staggered domains in the osculum of the adult sponge, and in the opening of the choanocyte chamber (Leininger et al., 2014). In *S. ciliatum* the choanocyte chamber is the module of axis determined by water flow.

Expression of *Wnt* in ostia, incurrent opening of aquiferous canals, is described in adult *Oscarella lobularis* (Homoscleromorpha). This sponge has a true epithelium, and ostia form by epithelial invagination of the expinacoderm. Localization of *Wnt* expression is characteristic of epithelial appendage formation in eumetazoans such as *Hydra* or *Nematostella* tentacles and wings in *Drosophila* (Lapébie et al., 2009).

The data on the diversity of Wnt pathway components, their phylogeny and expression allow us to make two important conclusions. (1) Sponges acquired their Wnt ligands independently of Cnidaria + Bilateria. It is still unclear whether the small number of ligands (two or three) in some species is a consequence of secondary loss or an ancestral condition. Lineage-specific expansion has definitely occurred in Calcarea. (2) Wnt pathway is likely to be involved in axes specification in Porifera. The sets of Wnt ligands expressed in a pole-dependent manner (the posterior half of the larva, the osculum, the opening of the choanocyte chamber). An opposite viewpoint is based on the study of *Wnt* expression in juvenile *E. muelleri* (Windsor Reid et al., 2018). It demonstrated the expression of *Wnt* ligands in different cell types not restricted to any region of the sponge. Although no expression of any *Wnt* in the osculum was shown, pharmacological alteration of this pathway increased the number of oscula upon activation and decreased upon inhibition (Windsor & Leys, 2010; Windsor Reid et al., 2018). So, we tend to think that in

sponges Wnt is implicated in axial patterning rather than cell specification, as the first metazoan mechanism of antero-posterior determination.

The second key player in axial patterning is the transforming growth factor (*TGF-beta*) signaling pathway. Core components of this pathway are extracellular ligands, transmembrane receptors and transcription factors SMADs. The superfamily of TGF-beta ligands encompasses many families of proteins, the main families being TGF-beta *sensu stricto*, BMPs, Nodals and Activins. Receptors are single pass serine-threonine kinases of two classes, TGFbRI or TGFbRII. Upon binding of the ligand, type II receptors activate type I receptors, and they phosphorylate SMADs, which leads to transcriptional activation. The patterning of the dorso-ventral axis during the development of Bilateria starts with the expression of BMP and Chordin (a soluble inhibitor of TGF-beta ligands) at the opposite sides of the embryo. At later stages TGF-beta signaling is involved in numerous developmental processes, from germ layer specification to the development of organs and tissues (Kitisin et al., 2007). This pathway is a novelty of Metazoa since neither ligand nor receptor molecules are found outside the animal kingdom (Srivastava et al., 2010).

A. queenslandica genome contains eight *TGF-beta* genes, of which two belong to TGF-beta *sensu stricto* and six fall into divergent clades such as GDF or DVR (Srivastava et al., 2010). Phylogenetic analysis of these diverged genes group suggests an independent expansion of ligands. There are ligand receptors of both types. The expression of one of the ligands is described in the development. Similarly to one of the *Wnt* genes, this *TGF-beta* is expressed in some micromeres from early cleavage stages. It is dynamically expressed throughout the outer cell layer of the embryo, and then in a concentric pattern around the posterior pole and at the anterior pole of embryo (Fig. 7C). In contrast to bilaterians and anthozoans, where TGF-beta patterns the axis perpendicular to the one patterned by Wnt signals, in *A. queenslandica* Wnt and TGF-beta are expressed along the same embryonic axis (Adamska et al., 2007).

A calcisponge *S. ciliatum* has 22 *TGF-beta* genes, one of which falls into TGF-beta *sensu stricto* family and remaining sequences cannot be ascribed to any defined eumetazoan families (Leininger et al., 2014). Several ligands are expressed uniformly in oocytes and during cleavage, while the expression of four *TGFs* is restricted to the macromeres located at the posterior pole of the embryo. The most prominent region of *TGF-*

beta expression in the adult sponge is the apical region: seven ligands are expressed at a significantly higher level in the apical part of sponge bearing the osculum (Fig. 7D). Thus, the expression of TGFs is similar to that of Wnt in *S. ciliatum*, being predominantly posterior in the larvae and apical in the adults. *SMAD* genes are expressed either in the same cells or in the cells adjacent to those expressing *TGF-beta* ligands. In contrast to ligands, downstream components of pathway are expressed more uniformly along the apico-basal axis of the adult sponge.

A search in *H. dujardini* transcriptome revealed eight TGF-beta ligands. All of them have a structure typical of TGF-beta: a signaling peptide at the N-terminal end, a large latency-associated peptide, the site of protease furin cleavage, and the C-terminal signal peptide with cysteines in conserved positions. Phylogenetic analysis revealed some similarities with other sponges: three sequences fall into the TGF-beta family *sensu stricto*, while the other five ligands do not belong to any eumetazoan families (Borisenko et al., in press). Interestingly, TGF-beta s.s. ligands have only been identified in deuterostomes and ctenophores, with no members found in the genome screens of *C. elegans*, *Drosophila* or *Nematostella* (Matus et al., 2006; Pang, Ryan, Baxevanis, & Martindale, 2011; Srivastava et al., 2010).

Many developmental processes in Bilateria involve *Hedgehog pathway* in a classical morphogen mode, acting in a concentration-dependent manner (Jiang & Hui, 2008). The Hedgehog ligand, a secreted protein, undergoes autocatalytic cleavage following acetylation and binds to Patched receptor on the cell surface. Hedgehog-competent signal cells express a zinc finger transcription factor Cubitus interruptus (Ci), which undergoes cleavage and releases a peptide (CiR) translocating in the nucleus and acting as a transcriptional repressor. The binding of Hedgehog to Patched allows the suppression of Ci cleavage; a full-size Ci works as a transcriptional activator. Thus, the level and the set of expressed target genes depend on the ligand concentration. In this way Hedgehog participates in many patterning processes such as limb formation in vertebrates and the establishment of segment polarity in *Drosophila*. However, in some cases this pathway acts as short-range signaling, e.g., in case of the induction of the neural floor plate by the notochord in vertebrates.

Hedgehog genes have not been identified outside cnidarians and bilaterians but a Hedgling homolog was found in a choanoflagellate *Mono-siga brevicolis* (King et al., 2008). The expression of two Hedgehog

genes has been analyzed in *Nematostella vectensis* (Matus, Magie, Pang, Martindale, & Thomsen, 2008). One of them is expressed in the pharyngeal ectoderm, while the other one is expressed in the endoderm; the receptor Patched is expressed only in the endoderm. This pattern suggests that both short- and long-range Hedgehog-mediated pathways may function in Cnidaria. Neither the ligand nor any other key components of this pathway were found in the ctenophore *Mnemiopsis leidyi* (Ryan et al., 2013). As at least some components of this pathway are present in choanoflagellates, its loss in Ctenophora seems to have been secondary and independent (Adamska, 2015).

A typical Hedgehog protein contains an N-terminal Hedge domain and a C-terminal Hog domain. Hog domain separates due to autocatalytic cleavage. Both these domains are found in *A. queenslandica* genome but in different genes. The key domain, Hedge, is a part of a large transmembrane protein Hedgling also containing the von Willebrand domain, cadherin repeats, immunoglobulin and EGF domains. *Hedgling* expresses during late embryonic development and is associated with the formation of the sensory pigment ring at the posterior pole of the embryo. It is unknown whether Hedgling proteins undergo modifications and/or cleavage. If they do not, this transmembrane protein could only signal across adjacent cells through direct contact of cell surfaces, in contrast to the true Hedgehog. The genome of *A. queenslandica* contains several other components of the Hedgehog pathway but not the receptor Patched (Richards & Degnan, 2009; Srivastava et al., 2010). It is unclear whether Hedgling transmits intracellular signals through the canonical Hedgehog pathway or through an unidentified mechanism (Adamska et al., 2007).

Growth factors family encompasses *fibroblast growth factors (FGF)*, platelet-derived growth factors (PDGF), vascular endothelial growth factors (VEGF) and epidermal growth factors (EGF). All of these proteins were described as growth factors for defined cell types and act through the same type of receptors, although ligands do not demonstrate similarity of sequences. FGF bind to receptor tyrosine kinase that initiates a series of phosphorylation events, the tyrosine kinase cascade, resulting in activation of transcription factors. Unlike other signalings, there is no exact transcription factor activated by FGF pathway, and the output of the pathway activation depends on the combination of ligand, receptor and cellular content.

FGF signaling is involved in various developmental processes in Bilateria, from axes specification to tissue induction and organ patterning (Thisse & Thisse, 2005). Another conserved role of FGF is boundary formation as shown in *Hydra* (Sudhop et al., 2004). Nevertheless, a true FGF protein is the novelty of Bilateria + Cnidaria: no FGF ligand was found in *Trichoplax*, *A. queenslandica*, *Mnemiopsis* or *Monosiga* genomes although FGF-like proteins with a similar domain organization were identified in many Metazoa including sponges (King et al., 2008; Ryan et al., 2013; Srivastava et al., 2008, 2010). Receptor tyrosine kinases are broadly distributed from Protista to Bilateria, and some of them display a weak similarity to the intracellular portion of the FGF receptors (Manning, Young, Miller, & Zhai, 2008). The intracellular signal transduction proteins are all present in *Monosiga*. This demonstrates a gradual assembly of the pathway, from signal transduction cascade utilized by single cell eukaryotes, through addition of receptors allowing more sophisticated sensing of the neighboring cells in the colony, to the emergence of a highly effective morphogen in the ancestor of Cnidaria + Bilateria (Adamska, 2015).

All signaling events mentioned above are mediated by secreted soluble proteins. In the *Delta/Notch signaling* pathway both the ligand (Delta or Serrate) and the receptor (Notch) are transmembrane proteins, and the signaling event means close contact of two neighbor cells. Upon binding of the ligand to the receptor, several proteolytic modifications occur at the ligand-receptor complex resulting in the production of Notch intracellular domain, which is translocated to the nucleus and acts as a transcription activator. This pathway appears to be the evolutionarily the oldest one: all the participants are identified in *Monosiga* genome except ligands. In choanoflagellates Delta/Notch might be involved in sensing environmental stimuli rather than intercellular signaling (Richards & Degnan, 2009).

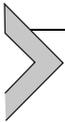
Delta/Notch signaling is implicated in the development of many organs in Bilateria, and is crucial for segmentation in Vertebrata (Andersson, Sandberg, & Lendahl, 2011). In Cnidaria it is involved in the boundary formation during budding and affects oogenesis and the differentiation of nematoblasts and neurons (Käsbauer et al., 2007; Marlow, Roettinger, Boekhout, & Martindale, 2012; Matveev, Shaposhnikova, & Podgornaya, 2007; Münder et al., 2010). In sponges, Delta/Notch pathway components and their developmental expression

are described in *A. queenslandica* (Gazave et al., 2009; Richards & Degnan, 2012; Richards et al., 2008). Five different Delta ligands, one Notch receptor and all core components were identified. The expression of *Notch* and *Delta* genes indicates that they are involved in specification of putative sensory cells, with *Notch* expression being broad and the five *Delta* genes displaying dynamic, unique patterns. The domain composition of the identified genes, as well as their expression patterns, indicate that the pathway operates in a manner similar to that in Cnidaria + Bilateria, and is involved in evolutionarily conserved processes such as cell-type specification and differentiation.

Nitric oxide (NO) pathway is another signaling mechanism usually mentioned in the context of blood vessel physiology or differentiation of endothelial cells. NO is an ancient signal molecule working *via* the activation of its primary cytoplasmic receptor, soluble guanylyl cyclase, or by direct covalent modification of target proteins such as *S*-nitrosylation and nitration. Data about its participation in early development are just beginning to accumulate although the role of NO in metamorphosis has been described for many phyla of invertebrates (Annona et al., 2017; Tomankova, Abaffy, & Sindelka, 2017).

Sponges have an almost full repertoire of neuronal genes needed to form functional neural scaffolds (Francis et al., 2017; Sakarya et al., 2007). Nevertheless, they have no neuronal structures. This is especially strange in case of sponge larvae, which have no neurons but are photosensitive and can coordinate ciliary beating for directional driving. It was elegantly demonstrated that NO is an activator of larva metamorphosis in *A. queenslandica* (Ueda et al., 2016). An inhibition of the NO pathway suppresses metamorphosis whereas an addition of endogenous NO activates it. In this case NO pathway works upstream of (or together with) ERK (extracellular signal-regulated *kinase*) pathway, one of the many receptor tyrosine kinase cascades, and the expression of NO synthase increases in localized cell populations when the larva becomes competent.

We can see that metazoans have an almost full toolkit for body patterning and the establishment of different cell types. Until new data about intercellular communication in sponges have been obtained, we can put forward two main questions regarding metamorphosis: (1) which of the signaling pathways and in what hierarchical order are involved in metamorphosis? (2) How is the body axis determined after metamorphosis and is it the same axis as in the larva?



7. CONCLUSIONS AND PERSPECTIVE

During metamorphosis sponge larvae lose ciliary cells and this ability to locomotion. The similarity of larval ciliated cells with the choanocytes of the adult sponge gave rise to the classical hypothesis of the “inversion of the germ layers” in sponges (Delage, 1892). Now that we have evidence from different sponges, we know that the fate of ciliated cells is species-specific. At the same time, ciliated cells often leave their place in the epithelial-like sheath and migrate inside during metamorphosis, *i.e.*, undergo EMT. Here we tried to show the connection between this process and the signaling pathways present in sponges through molecules involved in intercellular junctions. While proteins managing EMT were not found in transcriptomes of adults or larvae of *H. dujardini*, the this process in sponges remains mysterious. At the same time, sponges have most of the components of the six main signaling pathways. There is evidence that in sponges Wnt and TGF-beta signaling pathways are implicated in axial patterning rather than cell specification, and these pathways were the first metazoan mechanism of antero-posterior determination. The link between the axis of larvae and adult sponge looks highly likely as many signaling molecules are expressed at the posterior larval pole and in the osculum of the adult sponge. However, it is still unclear whether expression of signaling molecules in adult sponge marks the axis heredity from the larva, or it is *de novo* established after metamorphosis. The developmental regulation pathways may be traced with the help of transcriptomes of sponge development stages, which are available only for a couple of species. The application of traditional techniques to simple animals at the key root position of Metazoa is a necessary step for understanding developmental events at the molecular level.

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