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Review Article

Extracellular matrix and morphogenesis in cnidarians: a tightly knit relationship

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Cnidarians, members of an early-branching metazoan phylum, possess an extracellular matrix (ECM) between their two epithelial cell layers, called the mesoglea. The cnidarian ECM, which is best studied in *Hydra*, contains matrix components reflective of both interstitial matrix and basement membrane. The identification of core matrisome components in cnidarian genomes has led to the notion that the basic composition of vertebrate ECM is of highly conserved nature and can be traced back to pre-bilaterians. While in vertebrate classes ECM factors have often diverged and acquired specialized functions in the context of organ development, cnidarians with their simple body plan retained direct links between ECM and morphogenesis. Recent advances in genetic manipulation techniques have provided tools for systematically studying cnidarian ECM function in body axis patterning and regeneration.

Introduction

The extracellular matrix (ECM) constitutes a complex glycoprotein network that serves as structural support for tissues, in particular for epithelial cell layers that are organized by and aligned on basement membranes (BMs). The ECM not only forms a scaffold for cell attachment and migration, but it also provides important signaling cues directly through adhesion receptors like integrins [1], or by controlling the diffusion, accessibility, and turnover of soluble growth factors [2]. ECM evolution is tightly linked to the emergence of multi-cellularity, which in animals produced the highest complexity of cell types and body plans [3]. The matrisome of basal metazoan animals is therefore part of the early genetic toolkit controlling animal development. Essential components of this ancient gene repertoire are homeobox (Hox) transcription factors [4] and molecules of the wingless/integrated (Wnt) signaling pathway [5]. These conserved developmental regulators pattern the primary, anterior–posterior axis in bilaterian animals [6] (e.g. vertebrates, arthropods), but recent evidence indicates that, in addition to the well-described specification of the oral region by Wnts [7], also Hox factors have deep evolutionary roots in pre-bilaterians, namely cnidarians [8–11]. The connection between signaling and ECM in these processes is an emerging field in cnidarian research and promises important implications for animal morphogenesis in general.

Cnidarians (corals, sea anemones, jellyfish, and hydroids) (Figure 1) are commonly regarded as the first animal phylum that evolved a complex nervous system [12] and a true epithelial organization [13], although there is increasing evidence for functional epithelia in sponges [14,15]. They are an early-branching metazoan phylum that diverged from bilaterians more than 600 million years ago and contains ∼9000 mostly marine species [16]. Cnidarians are characterized by a diploblastic, radial symmetric body plan with a single body axis (oral-aboral) (Figure 2A), although internal structures such as the gastric mesenteries show a morphological asymmetry that is reminiscent of bilaterians [9,11]. While anthozoans generally produce sessile polyps from the larval stage, medusozoans have in addition adopted the medusa (the common "jellyfish") as a free-swimming, often exclusively sexual form, emanating from the polyp. During the last years, cnidarian model organisms as the hydrozoan *Hydra* and the anthozoan starlet sea anemone *Nematostella vectensis* have been instrumental in developmental studies targeting fundamental questions of body axis evolution and patterning. For both organisms genome and single-cell

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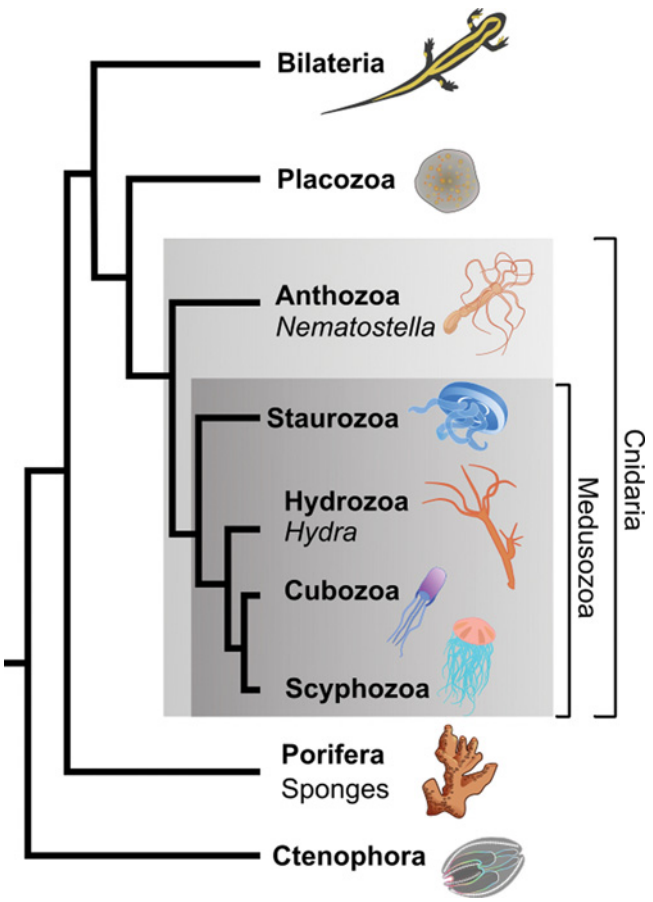

Figure 1. Cnidarian phylogeny

Evolutionary relationships among bilaterians (protostomes and deuterostomes), comb jellies (ctenophora), placozoa, sponges and cnidarians. The five cnidarian classes comprise Anthozoa, Hydrozoa, Scyphozoa, Cubozoa, and the recently defined Staurozoa [86]. Major research model systems are *Hydra magnipapillata* [17] and *Nematostella vectensis* [18].

RNA-Seq data are available [17–19] and recent advances in transgenesis [20,21] and genetic knockdown and knockout techniques [22,23] have revolutionized the field. This review summarizes the knowledge on the cnidarian ECM exemplary for the model organism *Hydra* and highlights the critical involvement of ECM factors in morphogenesis and regeneration.

Structure, composition, and dynamics of the mesoglea

The body plan of *Hydra*, which is exemplary for the cnidarian polyp stage, comprises an elongated gastrula-shaped tube with a single opening at the oral end (Figure 2A). The mouth area, or hypostome, is surrounded by a ring of tentacles that harbor the cnidarian-specific stinging organelles, the cnidocytes [24]. Unlike most medusozoans, *Hydra* does not form a larva or free-swimming medusa [25]. The polyp usually propagates asexually by budding but can also enter a sexual cycle. As all members of the cnidarian clade, hydras possess an ECM between their two epithelial cell layers, the outer ectodermis and the inner endodermis, called the mesoglea [26,27] (Figure 2A). In longitudinal sections of the gastric region the mesoglea appears as a thin, transparent line separating the two tissue layers (Figure 2B). Ultrastructural studies characterize it as a sheet-like elastic structure, ranging between 0.5 and 2 μm in width, with multiple pores that probably serve as cell–cell contact areas [28]. The mesoglea is generally organized from an amorphous basic structure with interspersed collagen and fibrillin fibers [27,29] (Figure 2C,D). It is synthesized asymmetrically by the epithelial tissues resulting in a central interstitial matrix (IM) sandwiched by two sheets of BM [30,31]. The identification and molecular characterization of several ECM components including laminin [32,33], type IV collagen [34], fibrillar collagens [35,36], and metalloproteinases [37–40] were mainly performed by

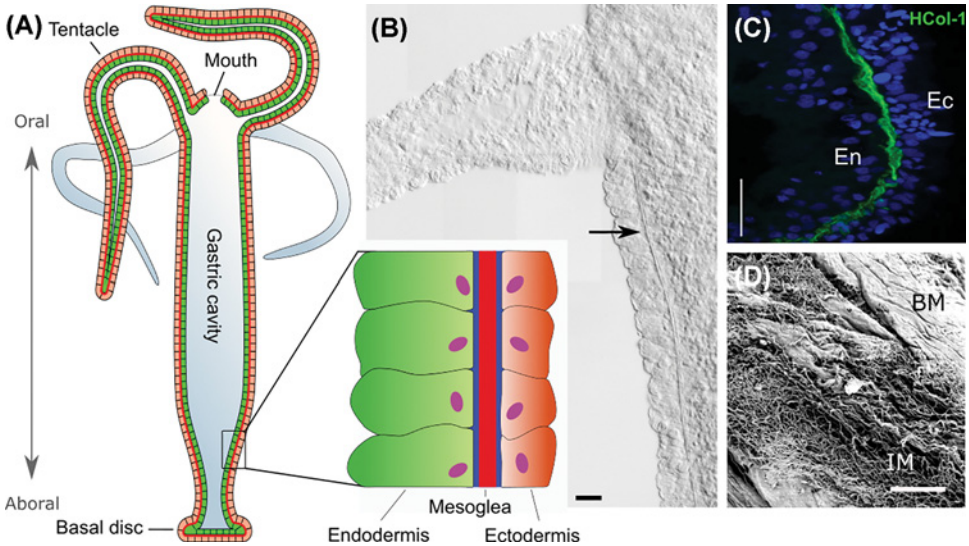

Figure 2. Schematic representation of the *Hydra* body plan

(**A**) *Hydra's* body forms a gastric tube with a foot region and a mouth opening at the tip of the head pole. This "hypostomal" region is surrounded by a ring of tentacles and harbors the head organizer. The entire body wall is organized as an epithelial bilayer with an inner endoderm and an outer ectoderm separated by an intervening ECM, the mesoglea. (**B**) Phase contrast image of the *Hydra* body wall. The mesoglea is visible as a fine transparent line (arrow). Scale bar = 50 μm. (**C**) A longitudinal cryosection stained with *Hydra* collagen 1 antibody [30] shows the central fibrous region (green) of the mesoglea. Cell nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). En, endoderm; Ec, ectoderm. Scale bar = 100 μm. (**D**) Scanning electron micrograph analysis of the isolated *Nematostella vectensis* mesoglea. The thin layer of BM is partially detached from the underlying fibrous IM. Scale bar = 10 μm. Figure 2C was provided by Jennifer Strompen.

Sarras and co-workers and confirmed the high conservation of core vertebrate ECM factors in metazoan evolution [27,28,32–39,41,42].

The mesoglea can be isolated in an intact form free of epithelial cells by a freeze/thaw procedure [43] and mesoglea components in *Hydra* have been visualized both in whole mounts and in isolated mesoglea samples [28]. Type IV collagen (Hcol4) antibodies stained dotted parallel lines along the oral–aboral axis that to a large extent were co-localized with laminin. The laminin staining in addition visualized intersecting perpendicular lines suggesting a network formation. The type I collagen (Hcol1) staining (Figure 2C) showed a grid of fibers with regularly distributed intervals of 2–3 μm. In addition to Hcol1, Zhang et al. have isolated a number of additional fibrillar collagens from *Hydra* and characterized their domain organization and expression patterns [36]. Most of the *Hydra* collagens belong to the A clade collagen chains and exhibit unusual N-propeptide domains like whey acidic protein (WAP) domains. In Hcol6, the collagen sequences are interspersed by several von Willebrand factor (vWF) A domains, indicating a combination of collagen and vWF A domains already in the diploblastic lineage. A recent proteomic analysis of isolated *Hydra* mesoglea confirmed the presence of previously described ECM factors and identified additional constituents as the matricellular Thrombospondin (HmTSP), which interestingly was characterized as a novel Wnt signaling modulator (see below) [23].

In steady-state *Hydra* polyps, the epithelial stem cells of the body column constantly proliferate causing a tissue flow from the mid-gastric region toward the tentacles and basal disc where terminal differentiation takes place [44,45]. Antibody labeling experiments of Hcol1 and laminin in live animals performed by Aufschnaiter et al. [30] for the first time allowed tracking of local mesoglea structures during a full cycle of cell division and displacement along the body axis of the polyp. Until then it was believed that the mesoglea functions as a stationary scaffold on which the epithelial cells actively migrate. Remarkably, the mesoglea was shown to move in parallel to the epithelial cells to a significant degree and to be similarly displaced at basal disc and tentacle tips (Figure 3). New mesoglea is constantly produced in the upper region of the body column. During budding, the mesoglea of the parental animal is initially stretched into the evaginating bud, followed by turnover and re-synthesis of ECM components [30]. The fibrillar collagen content was dramatically reduced during most phases of bud formation, probably resulting in a more elastic ECM structure during morphogenesis. Thus, the mesoglea in *Hydra* can be described as a highly dynamic structure

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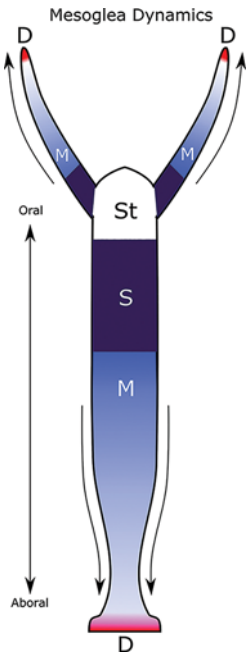

Figure 3. Mesoglea dynamics in *Hydra*

Mesoglea is constantly synthesized in the upper part of the body column (S, dark blue) and at the tentacle bases. Graded blue areas indicate mesoglea movement (M) toward tentacle tips and peduncle where it is degraded (D, red). The mesoglea in the head region is stationary (St, white). The scheme is adapted from Aufschnaiter and co-workers [28].

that is integrated into the global pattern of tissue regeneration, movement and turnover, reminiscent of connective tissue behavior during vertebrate gastrulation [46,47]. A notable exception is the head region, which harbors the Wnt-expressing cells of the hypostomal organizer [48]. The mesoglea of this area remains stationary and head tissue lysates showed low proteolytic activity for ECM proteins [30]. The proliferating epithelial cells of the head move relative to the mesoglea toward the hypostomal tip and tentacle bases [44]. This asymmetric dynamic could imply a role for the ECM as a "corset" for the fine-tuned signaling that establishes and maintains the head structure by local gradients of different Wnt ligands [49].

A hierarchy of ECM factors activated during head regeneration

Hydra has the remarkable ability to reorganize into its full structure from dissociated single cells within 96 h, a process that includes the re-establishment of the polarized epithelia and the formation of *de novo* head organizing centers [50,51]. Sarras et al. have shown that during reaggregation mesoglea components are deposited between the epithelial bilayer by approximately 12–17 h of aggregate formation and their translation rate peaks by 48–72 h of development [52]. Ultrastructural data indicated that a mature mesoglea is formed by 48–96 h of aggregate formation. Inhibitors of collagen cross-linking and proteoglycan formation reversibly blocked the development of *Hydra* cell aggregates indicating an essential role of mesoglea components for morphogenesis [52]. Cutting of hydras into two halves induces a full regeneration of the respective missing part within 36–48 h (Figure 4) [53]. A recent combined RNA-Seq and SILAC (stable isotope labeling by/with amino acids in cell culture) proteome analysis of *Hydra* head regeneration indicated staged molecular events during this process: a fast injury response at 0.5 h followed by a pre-patterning and remodeling phase of regenerating tissue up to 12 h and a late differentiation phase of the new head structure [54]. The process of head regeneration is dominated by several major signaling pathways including C-Jun N-terminal kinase (JUNK)/extracellular signal-related kinase (ERK)/reactive oxygen species (ROS), which regulate the early wound healing response [55] and Wnt/β-Catenin signaling that is up-regulated during the late patterning and cell proliferation phase. How are ECM factors involved in these different stages of body regeneration? Shimizu et al. reported that the mesoglea retracts and is not detectable at the head pole 1 h after decapitation with the cells of the fused epithelial

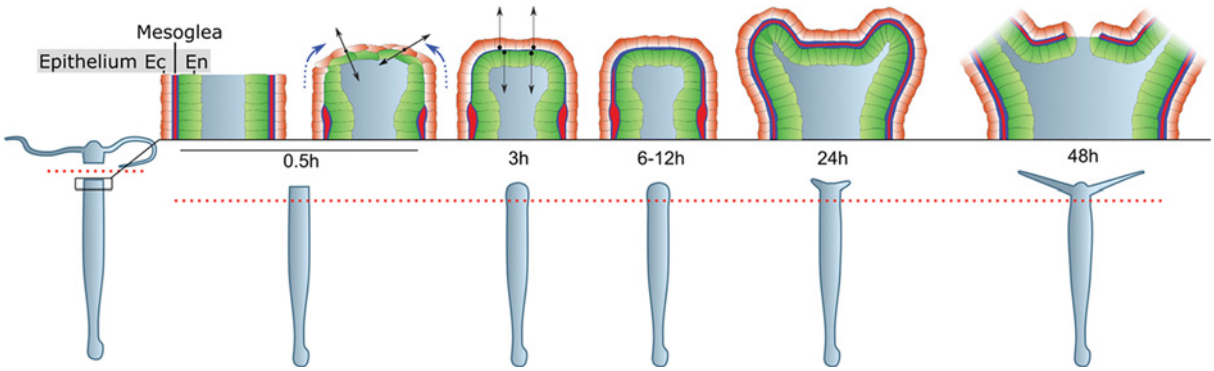

Figure 4. *Hydra* head regeneration

Head regeneration is initiated by amputation of the head at approximately 80% of body length. Wound closure within the first 30 min is a morphollactic process during which migrating epithelial cells from the remaining body wall acquire a stretched, depolarized morphology. During this phase the mesoglea retracts from the wound site. The epithelium is then reorganized during the first 12 h of regeneration, accompanied by BM *de novo* synthesis. Morphogenesis of the new head structure and full mesoglea re-synthesis takes place in the late phase of regeneration during 12–48 h.

tissue showing a stretched, non-cuboidal morphology [56] (Figure 4). Studies both in *Hydra* and *Nematostella* indicate an immediate activity of matrix metalloproteases at the wound site indicative of local tissue reorganization and mesoglea resorption [54,56,57]. A comparative analysis of laminin β1 and Hcol1 mRNA and protein up-regulation during head regeneration using whole mount *in situ* hybridization or immunofluorescence was not able to resolve a temporal hierarchy in gene expression. However, the BM-associated laminin preceded fibrillar collagen detection by 8 h at the protein level [56].

When we mapped the expression patterns of prominent mesoglea genes using available RNA-Seq data [54] for several time points of head regeneration, a global hierarchy between BM and IM factors in the regenerating head tip was clearly evident (Figure 5). While BM factors (Laminin, Collagen type IV, Perlecan) got up-regulated gradually after head amputation, IM-associated genes were generally not increased in their expression levels before the pre-patterning phase at 12 h. An exception was Hcol6, which followed the dynamics of the BM-related genes. This might indicate that Hcol6, which is supposed to be a network-forming collagen that is distinctly up-regulated during budding [36] might be physically associated with the BM. Interestingly, BM *de novo* synthesis closely followed the dynamics of Wnt3/β-Catenin that peaked at 24 h. These data suggest local epithelial reorganization and morphogenesis concomitant with BM synthesis as a prerequisite of mesoglea deposition and further head development, which is initiated at approximately 12 h. In line with this, Shimizu et al. have shown that knockdown of laminin β1 inhibits head regeneration and fibrillar collagen deposition [56]. Collagen type IV stands out as the most prominently expressed ECM factor during re-formation of the *Hydra* body axis and head organizer (Figure 5). Interestingly, it also functions as an essential scaffold for BM assembly during mammalian embryonal development [58]. Recent findings clearly underscore its primordial role for the evolution of multi-cellularity in general [59,60] and several excellent reviews have highlighted the conserved function of the BM for tissue repair, cell polarity regulation, and the formation of growth factor gradients [2,14,61–63]. A direct relationship between epithelial cells that have to re-adjust their apico-basal orientation and the newly formed BM might be the basis of the morphollactic regeneration process in *Hydra* (Figure 2). In contrast with epimorphosis morphollaxis represents a regeneration mode that occurs in the absence of cell proliferation and does not lead to blastema formation [64]. The remaining part in this case undergoes dramatic remodeling of the pre-existing tissue, which acquires higher motility and altered cell shapes. The early up-regulation of matrix metalloproteases as *Hydra* matrix metalloprotease-1 (HMMP-1) during head regeneration underscores this notion (Figure 5).

In their review on ECM dynamics and morphogenesis, Daley & Yamada have summarized several experimental findings in *Drosophila* and mammals that address related processes in organ development [65]. A particularly striking example is branching morphogenesis in the mammalian salivary gland or lung. A localized deposition of ECM here creates anisotropic force fields that induce cleft formation and promote epithelial tissue expansion [66–68]. Snail transcription factors that are well-described mediators of epithelial mesenchymal transition, a developmental process which involves loss of cell polarity and adhesion, down-regulation of Cadherin and increased cell motility, are transiently expressed at epithelial branch sites [69]. Interestingly, the expression of the *Hydra* Snail ortholog peaked in

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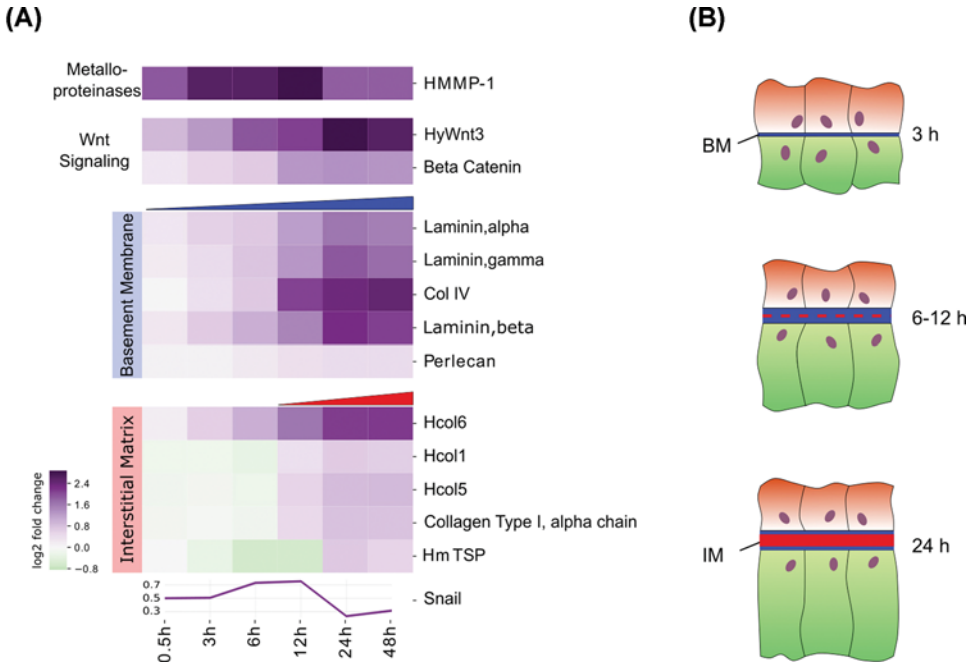

Figure 5. Transcriptome of mesoglea regeneration

(**A**) Heat map showing the dynamics of transcript levels for major mesoglea components confirmed by proteomic analysis presented in Lommel et al. [23]. *HMMP-1* and *Snail* were included in addition to characterize the dynamics of mesoglea turnover and cellular reorientation, respectively. Only components that were significantly differentially expressed ($P<0.05$) at one or more time points are shown. The analysis is based on head regeneration transcriptome data published in Petersen et al. [54]. The expression of BM factors precedes the re-synthesis of IM mesoglea components during head regeneration as illustrated in (**B**). For clarity reasons Snail expression is presented as a graph and shown to be transiently increased during BM re-synthesis. Transcript IDs were obtained by BLAST searches of the *Hydra* head regeneration transcriptome assemblies on https://research.nhgri.nih.gov/hydra. (HMMP-1: comp24947_c0_seq1; HmTSP: comp24178_c0_seq1; HCol-1: comp28519_c0_seq1; HCol-5: comp18600_c0_seq1; HCol-6: comp25324_c0_seq1; Collagen type I, α chain-1: comp21769_c0_seq1; Perlecan: comp27178_c0_seq1; Laminin, β: comp25312_c0_seq1; Col IV: comp20380_c0_seq1; Laminin, α: comp27401_c0_seq1; Laminin, γ: comp26030_c0_seq1; β-Catenin: comp12447_c0_seq1; HyWnt3: comp17763_c0_seq1; Hydra_Snail: comp21268_c0_seq1).

the regenerating head tip between 3 and 12 h (Figure 5), suggesting a possible cooperative interplay between BM and epithelial cell dynamics at the newly forming hypostome and tentacle bud structures. Taken together, the *de novo* patterning observed in regeneration processes of *Hydra* and other cnidarians most likely involve conserved ECM-dependent morphogenetic programs that in bilaterians were adopted for localized epithelial tissue reorganization.

ECM modulators of Wnt-induced axis formation

Wnt signaling is the major molecular system inducing axis formation in *Hydra* [48,70,71]. Canonical Wnt factors are continuously expressed from the blastoporal organizer creating an assumed β-Catenin activity gradient that provides positional information to the head and body tissues. This process recapitulates the formation of morphogenetic fields in the developing embryo [72] and requires an interplay with secreted antagonists like Dickkopf-1/2/4 [73].

To date, only few invertebrate ECM components have been described, which directly influence Wnt signaling. Prominent examples are glypicans, a family of heparan sulfate proteoglycans. Glypicans were extensively studied in *Drosophila* (Homologs *Dally* and *Dally-like*) and function in shaping the wingless morphogen gradient [74] by stabilizing ligand-receptor complexes [75]. Adversely, glypicans can also function in Wnt down-regulation by promoting the activity of the Wnt antagonist Notum [76]. The presence of heparan sulfate chains is often essential for the developmental role of glypicans [77]. Bause et al. have shown that morpholino knockdown of orally expressed *glypican1/2/4/6* in *Nematostella* embryos phenocopies knockdown of the Wnt receptor NvFrizzled5/8 and reduces oral markers [78]. A similar effect was observed upon treatment with sodium chlorate, an inhibitor of glycosaminoglycan

sulfation, indicating a conserved function of sulfated glypicans in regulating Wnt signaling during axial patterning. The authors hypothesized that glypican might increase Wnt ligand diffusion toward the Frizzled expression domain in line with findings in the vertebrate system showing that HSPGs maintain the solubility and activity of Wnts [79].

Recently, we have characterized *Hydra* Thrombospondin (HmTSP) as a novel β-catenin-regulated organizer gene [23]. TSPs are matricellular glycoproteins constituting a gene family of five members that form trimeric (subgroup A, TSP1 and TSP2) or pentameric (subgroup B, TSP3-5) oligomers [80]. Mammalian TSPs are multi-facetted regulators of tissue homeostasis and remodeling. They interact in a pleiotropic manner with a number of cell surface receptors and ECM proteins such as collagen, laminin, and matrilin [81]. The TSP signature domain is a C-terminal structural unit comprising tandem EGF domains, several calcium-binding TSP type 3 repeats and, a C-terminal lectin domain [82]. A coiled-coil domain close to the laminin G-like N-terminal drives oligomerization to either trimers or pentamers. The single HmTSP, like *Drosophila* TSP [83], belongs to the pentameric subgroup A TSPs.

HmTSP shows a striking ectodermal expression at the tip of the polyp's hypostome, which constitutes the core of the head organizer [23]. Double *in situ* hybridization experiments confirmed that *HyWnt3* and *HmTSP* are expressed from the same ectodermal cells. A recently established robust method for siRNA gene knockdown in adult *Hydra* polyps by electroporation allowed a functional analysis of HmTSP in the context of Wnt signaling and patterning [84]. Hydras in which β-Catenin activity is systemically increased by treatment with the GSK3β inhibitor Alsterpaullone produce ectopic organizers along the body column [85]. *HmTSP* knockdown led to increased numbers of these organizing centers indicating a negative regulatory function for HmTSP in axis formation. Unpublished findings in our lab have confirmed a direct binding of *Hydra* Wnt3 to HmTSP, suggesting that this interaction might compete with receptor binding. The unexpected role of TSP as a modulator of canonical Wnt signaling in *Hydra* represents a relevant example for primordial functions of ECM in morphogenesis that in more complex animals are obscured by pleiotropy and specialization. Recent efforts to establish reliable siRNA knockdown and CRISPR/Cas9 (clustered regularly interspaced short palindromic repeats/CRISPR-associated) knockout strategies in *Hydra* and other cnidarian model systems will serve as a basis for systematically probing the involvement of conserved ECM factors in processes of morphogenesis and regeneration on the organismal scale.

Summary

- Cnidarians form a sister clade to all bilaterians and are the first animal phylum with a true epithelium and organized nervous system. Their ECM composition reflects the core matrisome of basal metazoan animals.
- The cnidarian mesoglea is a sheet-like structure connecting the outer ectoderm and inner endoderm. It contains a central fibrous zone of connective tissue with two subepithelial layers of BM.
- In the model organism *Hydra*, the mesoglea is a highly dynamic structure that mirrors the tissue flow and turnover of epithelial stem cells.
- Head regeneration in *Hydra* is accompanied by a hierarchical expression of ECM factors suggesting a primordial role of BM in tissue morphogenesis.
- Canonical Wnt signaling that dominates axial patterning in cnidarians is modulated at the ligand/receptor level by conserved ECM molecules.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contribution

S.Ö. conceived and wrote the article. B.G.B. designed and prepared figures, performed the transcript analysis (heat map) of ECM genes in *Hydra* head regeneration, and contributed to the final version of the manuscript.

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Abbreviations

BM, basement membrane; ECM, extracellular matrix; HMMP-1, *Hydra* matrix metalloprotease-1; Hox, homeobox; IM, interstitial matrix; TSP, Thrombospondin; vWF, von Willebrand factor; Wnt, wingless/integrated.

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