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Components, structure, biogenesis and function of the *Hydra* extracellular matrix in regeneration, pattern formation and cell differentiation

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ABSTRACT The body wall of *Hydra* is organized as an epithelial bilayer (ectoderm and endoderm) with an intervening extracellular matrix (ECM), termed mesoglea by early biologists. Morphological studies have determined that *Hydra* ECM is composed of two basal lamina layers positioned at the base of each epithelial layer with an intervening interstitial matrix. Molecular and biochemical analyses of *Hydra* ECM have established that it contains components similar to those seen in more complicated vertebrate species. These components include such macromolecules as laminin, type IV collagen, and various fibrillar collagens. These components are synthesized in a complicated manner involving cross-talk between the epithelial bilayer. Any perturbation to ECM biogenesis leads to a blockage in *Hydra* morphogenesis. Blockage in ECM/cell interactions in the adult polyp also leads to problems in epithelial transdifferentiation processes. In terms of biophysical parameters, *Hydra* ECM is highly flexible; a property that facilitates continuous movements along the organism's longitudinal and radial axis. This is in contrast to the more rigid matrices often found in vertebrates. The flexible nature of *Hydra* ECM can in part now be explained by the unique structure of the organism's type IV collagen and fibrillar collagens. This review will focus on *Hydra* ECM in regard to: 1) its general structure, 2) its molecular composition, 3) the biophysical basis for the flexible nature of *Hydra*'s ECM, 4) the relationship of the biogenesis of *Hydra* ECM to regeneration of body form, and 5) the functional role of *Hydra* ECM during pattern formation and cell differentiation.

KEY WORDS: *Hydra, extracellular matrix, ECM biogenesis, laminin, collagen, morphogenesis, development*

Introduction

The last comprehensive review of *Hydra* ECM was published over a decade ago (Sarras and Deutzmann, 2001). Because significant advances have been made since that publication, this review will update the reader on the advances made in this area of *Hydra* ECM biology and put these advances in context with our current understanding of matrix biology.

As a Cnidarian, *Hydra* arose early during metazoan evolution; approximately 580 million years ago before the divergence of protosomes and deuterostomes. As a member of Cnidaria, *Hydra* is the first of existing groups with defined epithelial cell layers (seen as a bilayer) with junctional complexes present at the apical pole of the cells. *Hydra* therefore has true epithelial tissues as compared to the more primitive Porifera (sponges) that are thought to have diverged before *Hydra*. *Hydra*'s general body plan is organized as a gastric tube with a mouth and tentacle ring at the head pole and a peduncle and basal disk at the foot pole (Fig. 1). The entire body wall of *Hydra* (from the tip of its tentacles to the base of its foot process) is structured as an epithelial bilayer with an intervening extracellular matrix (ECM) (Fig. 1). In the basal-lateral compartment between the epithelial cells are the interstitial cells (i-cells) that comprise such phenotypes as nerve cells, nematocytes, and gametes for sexual reproduction (Fig. 1).

It should be noted however, that the high regenerative capacity of *Hydra* is solely due to the epithelial cells because polyps lacking any i-cells are fully capable of complete body regeneration (they are unable to feed themselves; however, because of the lack of nerve cells) (Bode, 2011). Because of its simplified structure and

Abbreviations used in this paper: ECM, extracellular matrix.

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high regenerative capacity, *Hydra* has been used as a model for analysis of cell-ECM interactions during morphogenesis and cell differentiation. Over the last two decades, biochemical, and molecular studies have established that the ECM of *Hydra* is composed of a broad spectrum of matrix components reflective of that seen in more complicated vertebrate systems. These matrix molecules have been shown to have an important role in morphogenetic and cell differentiation processes in *Hydra*. Molecular and biochemical studies have also established the structural basis for the biophysical properties of *Hydra* ECM. These studies in combination with early ultrastructural analyses indicate that the ECM of *Hydra* is a highly flexible matrix with elastic properties, mainly based on the macromolecular structure of its collagens (type IV and fibrillar collagens).

This review will include a discussion of *Hydra* ECM in regard to 1) its general structure, 2) its molecular composition, 3) the biophysical basis for the flexible nature of *Hydra*'s ECM, 4) the relationship of the biogenesis of *Hydra* ECM to regeneration of body form, and 5) the functional role of *Hydra* ECM during pattern formation and cell differentiation in this simple invertebrate organism that diverged early during metazoan evolution.

The general structure of the *Hydra* ECM based on early and current studies

Early morphological and biochemical studies on the structure and composition of the Hydra ECM

Initial ultrastructural studies of *Hydra* ECM (mesoglea) identified a broad spectrum of structural components and described it as an amorphous matrix of low density containing fine fibrils ranging from 5-50 nm in diameter (Hess, 1957; Wood, 1961; Gauthier, 1963). Cellular processes from the bilayer extend into the ECM and form cell-cell contacts between the endoderm and ectoderm (Hess, 1957; Wood, 1961; Haynes *et al*., 1968; Shimizu, 2008)

Davis and Haynes (1968) provided a detailed analysis of the ultrastructure of *Hydra* ECM under conditions in which the polyp was relaxed or contracted. In agreement with previous studies, they reported that *Hydra* ECM was approximately 0.5 to 2.0 μm in diameter and noted it was thickest in the body column and thinnest in the tentacles. They also indicated that it had three structural components based on TEM analysis, namely, an amorphous ground substance, fibrils, and particulate material. The fibrils consisted of three types. The smallest fibrils were 5-9 nm in diameter and their density in the ECM varied depending on whether the polyps were in a relaxed or contracted state. During contraction, these fibrils were randomly arranged and more densely packed while in a relaxed state the fibrils were in a more orderly arrangement and less densely packed. A second type of fibril that was less frequent was a thicker banded fibril that ranged from 36-45 nm in diameter and had a periodicity of approximately 30 nm. The final class of fibrils consisted of short thin fibrils that formed bundles oriented perpendicular to the long axis of the polyp. These fibrils extend from the basal plasma membrane of the ectoderm cells into the ECM suggesting some type of linker function.

Biochemical studies by Barzansky and Lenhoff (1974) and Barzansky *et al.,* (1975) provided additional insight into the macromolecular composition of *Hydra* ECM. Utilizing a combination of techniques including gel chromatography, amino acid analysis and thin layer chromatography for sugar moiety analysis in conjunction with radiotracer precursor-labeling; they reported that *Hydra* ECM had biochemical characteristics similar to vertebrate basal lamina. They also concluded that the amino acid profiles suggested the presence of collagens in *Hydra* ECM. Experiments using lathyritic agents known to cause reduced cross-linking of collagens indicated a role for these macromolecules in *Hydra* morphogenesis (Barzansky and Lenhoff, 1974).

As a consequence of 1) the types of matrix components forming the supramolecular structure of *Hydra* ECM and 2) the unique structural features of these components, *Hydra* ECM exists as a highly flexible and elastic structure as compared to normal ECM of vertebrates. Also in contrast to vertebrate ECM, the collagens of *Hydra* ECM are more easily extracted due to a reduced amount of cross-linking as will be discussed later in the *Hydra* collagen sections (Deutzmann *et al*., 2000). The unique properties of *Hydra* ECM provide the necessary rigidity for maintenance of body shape while at the same time permitting sufficient plasticity to allow extensive but reversible shape changes along the longitudinal and radial axis. Studies by Shostak *et al.,* (1965) showed that when isolated *Hydra* ECM was stretched to twice its original length, it would retract to its original length when released from tension. In this regard, it was noted in the ultrastructural studies of Davis and Haynes (1968) that in a contracted state, the fibrils of the central fibrous zone become irregular and fold upon themselves. This is unusual for vertebrate fibrillar collagens, which tend to be normally inelastic.

For a period of 16 years no further structural studies on

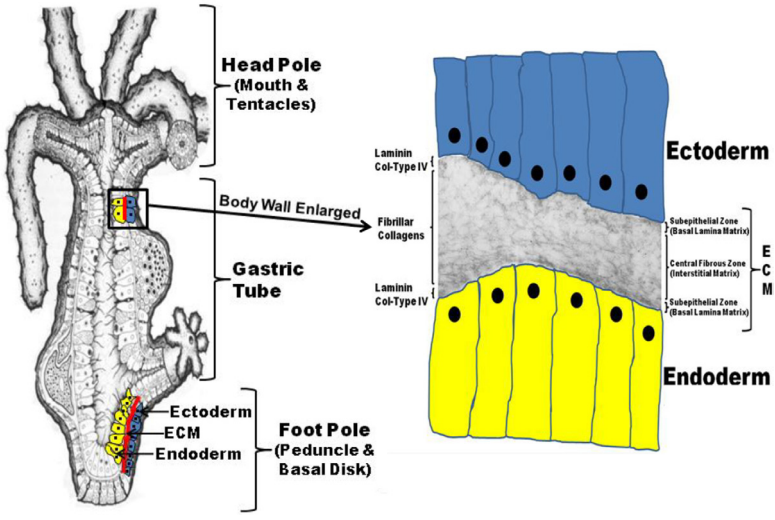

Fig. 1. The Hydra body plan is formed of an epithelial bilayer wih an intervening extracellular matrix (ECM). Hydra *exists as a gastric tube with a head process (mouth and tentacles) at the apical pole and a foot process (peduncle and basal disk) at the basal pole (left diagram). The entire body wall of* Hydra *(from the tip of the tentacles to the basal disk) is organized as an epithelial bilayer with an intervening ECM along the entire longitudinal axis of the organism.* Hydra*'s ECM is structured as two subepithelial zones (i.e. basal lamina matrix) with an intervening central fibrous zone (i.e. interstitial matrix). As shown in the composite diagram to the right that utilizes a transmission electron micrograph of* Hydra *ECM interposed between a drawing of two cell layers (ectoderm and endoderm),* Hydra *laminin and Type IV Collagen are localized to the two subepithelial zones of the matrix while* Hydra *fibrillar collagens (e.g. Hcol-I) are localized to the central fibrous zone or interstitial matrix.*

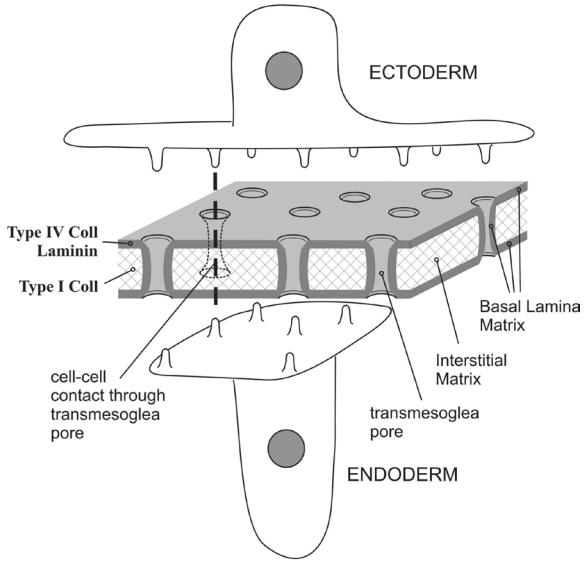

Fig. 2. Model of the *Hydra* extracellular matrix (ECM) structure showing cellular processes sheathed by a basal lamina and extending through the ECM from both the ectoderm and endoderm. *These processes allow for communication between the two cell layers through a cellular extension system that is sheathed by a basal lamina and thereby separated from direct contact with interstitial matrix components. Scheme taken from Shimizu et al. (2008), with permission.*

Hydra ECM were published. In 1991 the first of a series of papers were published that utilized a combination of biochemical, molecular, and cell biological approaches to clarify the macromolecular structure of *Hydra* ECM and to elucidate the role of cell-ECM interactions during cell differentiation and morphogenesis in this ancient metazoan. What follows, is an overview of what is currently known about the overall structure of *Hydra* ECM.

The Hydra ECM is a tri-laminar structure composed of two subepithelial basal lamina matrices and a central interstitial-like matrix

Beginning in the late 1980's experiments were begun to utilize *Hydra* as a developmental model to study the role of cell-ECM interactions during pattern formation in epithelial systems. The specific questions being asked related to the role of *Hydra* ECM in cell differentiation and morphogenetic processes. As a first step, it was essential to clarify the molecular composition and structure of *Hydra* ECM. The strategy implemented involved: 1) isolation of *Hydra* ECM and use of biochemical and immunological approaches to analyze the purified matrix preparation, 2) use of the purified *Hydra* ECM preparation to generate a battery of *Hydra*-specific polyclonal and monoclonal antibodies, 3) use of these *Hydra* specific antibodies as reagents to screen expression cDNA libraries and as probes to characterize the distribution of matrix components in *Hydra* ECM using morphological techniques, and 4) use of *Hydra*-specific antibodies and isolated matrix component domains as blocking reagents to study the role of cell-ECM interactions in *Hydra* using a number of regeneration bioassays. These functional studies were later complimented with antisense RNA studies to selectively knockdown *Hydra* ECM components during regenerative processes.

Initial biochemical and immunological studies using antibodies generated to vertebrate ECM components indicated that *Hydra* ECM had a spectrum of matrix components similar to those observed in more complicated invertebrates and in vertebrates. Specifically, evidence for the presence of collagen type IV, laminin, heparan sulfate proteoglycan and fibronectin-like molecules was presented (Sarras *et al.*, 1991). Pulse-chase autoradiographic studies in conjunction with translational and post-translational processing inhibitor studies supported the presence of collagen and proteoglycan components (Sarras *et al.*, 1991). Use of *Hydra*-specific monoclonal antibodies (Sarras *et al.*, 1993) in combination with special ultrastructural staining techniques (Sarras *et al.*, 1994) clarified that *Hydra* ECM contained distinct structural regions. Adjacent to the basal plasma membrane border of each epithelial layer was a defined basal lamina-like region named the subepithelial zone and intervening between these two basal lamina–like regions was a central fibrous zone that appeared similar to interstitial matrix. Subsequent cloning studies resolved this issue by showing that laminin chains were confined to the subepithelial zones (i.e. basal lamina) (Sarras *et al.*, 1994) and type I-like collagen was confined to the central fibrous zone (i.e. interstitial matrix) (Deutzmann *et al.*, 2000). Along with molecular analysis (Fowler *et al.*, 2000), monoclonal antibody to the NC1 domain of collagen type IV has recently localized this macromolecule with laminin to the two basal lamina layers adjacent to the ectoderm and endoderm (Shimizu, 2008).

Recently, confocal microscopy used to further localize laminin, *Hydra* collagen type IV (Hcol-IV), and *Hydra* fibrillar collagen type I (Hcol-I) within the ECM, has determined that *Hydra* ECM is porous with multiple trans-ECM pores ranging from 0.5 to 1 μm in diameter and about six pores per 100 mm^2 in density. Cellular processes from the ectoderm and endoderm, utilize these pores to form communications between the two layers within the ECM (Shimizu, 2008). The basal lamina components form a cylinder around these cellular processes as they pass through the ECM (Shimizu, 2008). The reader is referred to Fig. 2. for a diagram of this basal limina sheathed cell to cell communication system within *Hydra* ECM. Based on these studies, an overall structure of *Hydra* ECM is shown in Fig. 1. and a discussion of each of the major components (laminin, collagen type IV and fibrillar collagens) of *Hydra* ECM follows. A list of the major components of *Hydra* ECM with their general properties is shown in Table 1.

Molecular components of the *Hydra* ECM

Laminins

The laminin family of matrix proteins

Laminins represent a family of glycoproteins that are a major component of basement membranes (basal lamina). To date, at least sixteen different laminin heterotrimers have been identified in mammals and a number of laminins have been identified in invertebrates such as *Drosophila, C. elegans*, and sea urchin (Burgeson *et al.*, 1994; Richards *et al.*, 1994; Iivanainen *et al.*, 1995; Martin *et al.*, 1995; Miner *et al.*, 1995; Champliaud *et al.*, 1996; Benson *et al.*, 1999; Hutter *et al.*, 2000). Laminins are involved in basement membrane assembly and are an important component in the supramolecular architecture of matrix (Yurchenco and O'Rear, 1994; Yurchenco and Cheng, 1994; Timpl and Brown, 1996; Yurchenco, 2011). The heterotrimer isoforms are generated from multiple subunits to include the α, β, and γ chains. In vertebrates, we find many different α, β and γ chains and humans

have at least 16 heterotrimeric laminin species (Aumailley *et al.*, 2005). At least some of these chains may exist as alternatively spliced forms (Kallunki *et al.*, 1992; Galliano *et al.*, 1995; Talts *et al.*, 1999). ECM superstructure is based on assembly of a self-binding laminin mesh-like polymer, formation of a self-binding type IV collagen network polymer, and subsequent binding of various other ECM components to these networks (Colognato and Yurchenco, 2000). Interaction of laminin domains to cell surface ECM-binding proteins such as integrins and dystroglycan is involved with matrix assembly processes (Colognato *et al.*, 1999) and with cell signaling events (Darribere *et al.*, 2000; Wickstrom *et al.*, 2011; Yurchenco, 2011; Alexi *et al.*, 2011) associated with cell differentiation and morphogenesis.

Hydra laminin

Hydra laminin chains have been cloned and functionally analyzed (Sarras *et al.*, 1993; Sarras *et al.*, 1994). As stated, *Hydra* laminin is localized to the two subepithelial zones (basal lamina) of *Hydra* ECM. A partial sequence for a *Hydra* β1-like chain was initially reported (Sarras *et al.*, 1994). Studies have since been completed for description of the entire ORF of the α1 chain and partial description of a α-5-like chain (Zhang *et al.*, 2002). No γchain has thus far been identified, although the typical trimeric form for isolated *Hydra* laminin has been observed by rotary shadow TEM analysis (Zhang *et al.*, 2002). While *Hydra* laminin is localized to the two subepithelial zones (basal lamina) of *Hydra* ECM, it is synthesized exclusively by the endoderm, which means that the molecules have to diffuse through the mesoglea to reach the ectodermal layer (Sarras *et al.*, 1993; Sarras *et al.*, 1994). The location at the basal region of both cell layers suggests that laminin is required for proper cell function and differentiation. Consistent with this proposal, laminin secretion from the endoderm precedes secretion of *Hydra* collagen-I that arises from the ectoderm (Deutzmann *et al.*, 2000) and inhibition of laminin secretion through a RNA antisense technique will block collagen secretion (Shimizu *et al.,* 2002). Earlier studies have already established that antibodies to *Hydra* laminin will block *Hydra* morphogenesis (Sarras *et al.*, 1993) and other ECM-related processes such as cell migration (Zhang and Sarras, 1994).

While the mechanism(s) of signal transduction in *Hydra* is not fully known, some published data (Agbas and Sarras, 1994) and unpublished studies (Sarras laboratory) suggest the involvement of integrins, the primary class of ECM-receptors in higher animals (Darribere *et al.*, 2000; Wickstrom *et al.*, 2011). The *Hydra* ECM receptor data mainly pertains to a region in the short arm of the *Hydra* β1-like chain. In this regard, sequence analysis of the β1-like chain indicates the substitution of a FTGTQ sequence for the YIGSR receptor-binding sequence observed in vertebrates. Although the role of the YIGSR sequence in signal transduction-mediated processes has been questioned, published studies do indicate 1) its potential use as an inhibitor of human pre-B leukaemic cell growth and metastasis using SCID mice models (Yoshida *et al.*, 1999) and 2) its role in the guidance of axon growth cones (Hopker *et al.*, 1999). Such studies and others support its involvement in cell signaling processes.

More recent studies also validate the existence of the YIGSR ECM-receptor sequence (Saleh *et al.*, 2008). The substituted FTGTQ sequence in the *Hydra* β1-like chain has also been shown to interact with the cell surface under both in-vitro and in-vivo conditions (Sarras *et al.*, 1994) and affinity purification studies indicate that the FTGTQ sequence can interact with a *Hydra* integrin-like protein (Agbas and Sarras, 1994). Further analysis of 1) laminin-mediated cell signaling processes and 2) the role of laminin in the biogenesis and assembly of *Hydra* ECM is required to fully understand the relationship of pattern formation to ECM structure and assembly in this invertebrate organism. As an update, it is important to note that based on publication of the *Hydra* genome in 2010, it is now known that true integrin molecules are present in the genome of this organism (Chapman *et al.*, 2010). Based on this sequence data, more extensive structural and functional studies regarding Cell/ECM interaction can now be conducted.

Collagens

Collagens are found in all animals and are the most abundant protein of the extracellular matrix. The basic structure of this large protein family consists of multiple Gly-X-Y repeats. Extensive structural and functional diversity among collagens is accomplished by introducing interruptions in the triple helical domains and inclusion of various globular domains (Prockop and Kivirikko, 1995; Olsen and Niomiya, 1999; Ricard-Blum, 2011). Some collagens, like the basement membrane collagen type IV are found in all animals, whereas others are limited to particular groups. The fibrillar collagens for instance have previously only been found in vertebrates and have not been identified in invertebrates. In contrast, specialized

TABLE 1

MAJOR COMPONENTS OF *HYDRA* EXTRACELLULAR MATRIX

Basal Lamina ECM components	
Laminin	Contains α, β, and γ subunits in a trimeric cruciate (HLM) structure as viewed by rotary EM. Cloning studies indicate the alpha subunit is a vertebrate α5-like or *Drosophila*-like chain while the beta subunit is a vertebrate β1-like chain containing at least one defined cell binding domain (FTGTQ).
Collagen type IV (Hcol-IV)	A homotrimeric glycoprotein formed from three collagen IV α1-like chains. Each subunit contains a collagen domain at its N-terminus and a smaller non-collagenous domain (NC1) at its C-terminus. The collagen domain contains RGD cell binding motifs. TEM rotary shadow studies indicate that polymerization of Hcol-IV molecules within the ECM involves interaction of the NC1 domains. A typical 7S domain at the N-terminus appears not to be present in the mature ECM.
Interstitial ECM components (Fibrillar collagens)	
1) Fibrillar Collagens type I (Hcol1)	A homotrimeric glycoprotein formed from three collagen type I α1-like chains. Each chain contains an N-terminal propeptide and C-terminal propeptide. During processing the C-terminal pro-peptide is removed, but the N-terminal propeptide is retained in the mature molecule. This results in the formation of flexible fibrils but not thickened banded fibrils as typically seen in vertebrate type I collagens. Hcol-1's flexibility is enhanced by a reduction in its proline content and a loss of critical lysines involved in lysyl-cross bridging.
2) Hydra Collagen types 2, 3, 5, & 6 (Hcol 2; Hcol 3; Hcol 5 & Hcol 6)	*Hydra* fibrillar collagens Hcol 2, 3, 5, and 6 were characterized only from cDNA sequence analysis and subsequent computer modeling, and so unlike Hcol1 where the protein was also purified and biochemically analyzed, there is some degree of speculation about the *in vivo* nature of these proteins.

See the proposed structures of the fibrillar collagens in Fig. 3.

collagens such as the cuticle collagens *of C elegans* (Kramer, 1994) and the mini-collagens of *Hydra* nematocyst capsules (Kurz *et al.*, 1991) have only been found in invertebrates. Surprisingly, Kramer (1994) has reported that the cuticle collagens of *C. elegans* are encoded by more than a 100 genes. These observations indicate how diversity in collagen structure is utilized to meet the needs of a broad spectrum of specialized extracellular matrices. Accordingly, analysis of the structure of specialized collagens provides us with insight into the organization and function of ECM in both vertebrates and invertebrates.

While indirect evidence had suggested the existence of collagens in the ECM of *Hydra* (Hausman and Bernett, 1971; Barzansky and Lenhoff, 1974), more recent structural and functional analysis has provided a clear understanding of the types of collagens that exist in this invertebrate (Deutzmann *et al.*, 2000; Fowler *et al.*, 2000). These collagens include a basement membrane-type (*Hydra* collagen type IV, Hcol-IV) and an interstitial-type (*Hydra* fibrillar collagen, Hcol-1). Other *Hydra* fibrillar collagens have been more recently identified (Zhang *et al.*, 2007). As will be discussed, Hcol-IV and Hcol-1 collagens have been characterized at both the cDNA and protein level.

Hydra collagen type IV (Hcol-IV)

Collagen type IV is the second most prominent constituent of basement membranes after laminin. These collagens are glycoproteins composed of three subunits that form a polymerized network in conjunction with laminin (Hudson *et al.*, 1994; Borza *et al.*, 2000; Colognato and Yurchenco, 2000). Currently, six different types of collagen type IV subunits are known to exist in vertebrates (Yurchenco and O'Rear, 1994; Hopker *et al.*, 1999; Borza *et al.*, 2000; Vanacore R *et al.*., 2011). Invertebrate collagen type IV molecules have previously been identified in such organisms as *Drosophila* (Blumberg *et al.*, 1988), *C. elegans* (Guo *et al.*, 1991) and a number of other species (Kuehn, 1994).

As reported by Fowler *et al.* (2000) *Hydra* ECM also contains a collagen type IV. Analysis of the cDNA clone revealed a protein of 1723 amino acids, including an interrupted 1455 residue collagenous domain and a 228 residue carboxyl-terminal non-collagenous domain. Hcol-IV is similar to all known α(IV) chains, but again, most closely resembles vertebrate and invertebrate α1(IV) chains. Like *Hydra* fibrillar collagen, Hcol-IV also forms homotrimeric molecules. Electron microscopy reveals an irregular network of rod-like structures interrupted by globular domains. This network can be depolymerized by reducing agents to dimeric collagen molecules, joined via their C-terminal non-collagenous domains. Under extensive denaturing conditions, depolymerization can only be taken to the dimeric but not monomeric stage. This suggests that the individual polypeptide chains are quantitatively held together by non-reducible cross-links in addition to disulfide bonds. This behavior is quite different from the vertebrate collagen type IV that needs pepsin digestion for solubilization. For vertebrate collagen type IV, a model has been proposed in which four molecules aggregate via their N-terminal domains to form a spider-like structure. The interactions are stabilized via disulfide bonds and lysine derived cross-links, resulting in a highly protease resistant 7S domain. In addition, the C-terminal globular domain, NC1, binds to itself, mainly via disulfide bridges, to form a linear dimer.

Both interactions at the N-terminal and C-terminal ends lead to the proposal of an open network structure that can further polymerize via lateral aggregation of the triple helical domains (Yurchenco and O'Rear, 1994; Kuehn, 1994; Reinhart-King, 2011). In contrast, in *Hydra* while C-terminal interactions and lateral aggregation occurs, a stable 7S domain is not formed. A similar collagen type IV has also been reported in the worm, *Ascaris suum* (Noelken *et al.*, 1986). This form of collagen type IV favors a more flexible ECM then that seen in typical vertebrate matrices.

Hydra fibrillar collagens

1) Hydra fibrillar collagen 1 (Hcol-1). Fibrillar collagens make up the majority of matrix components within the interstitial matrix of the animal kingdom. Likewise, *Hydra* fibrillar collagens are the major component of *Hydra* ECM (Deutzmann *et al.*, 2000). The cDNA for the *Hydra* fibrillar collagen, Hcol-I, encodes a protein of 1412 amino acids. Hcol-I was the first fibrillar collagen to be identified in *Hydra*. The polypeptide isolated from *Hydra* ECM has an apparent molecular weight of 155 kDa. The subunit chains of Hcol-I form homotrimeric molecules that constitute the majority of the fibrils within the central fibrous zone (interstitial matrix). Sequence comparisons clearly define Hcol-I as a fibrillar collagen. The highest similarity is to the α chains of vertebrate collagens type I and II. A similar degree of similarity is found between Hcol-I and invertebrate sea urchin collagen (Exposito and Garrone, 1990) and a sponge fragment (Exposito *et al.*, 1992). Corresponding to the similarity at the sequence level, Hcol-I also exhibits the characteristic domain structure of fibrillar collagens, consisting of a central triple helical domain flanked by an N-terminal propeptide-like domain and a C-terminal propeptide (Fig. 3). It is note worthy that the triple helical domain with 340 uninterrupted GLY-X-Y repeats has exactly the same length as the fibrillar collagens of vertebrates, suggesting similar fibril forming possibilities.

Despite marked similarities in the primary structure, there are distinct differences in the supramolecular organization of vertebrate fibrillar collagen networks as compared to that seen in *Hydra* ECM. Hcol-I forms a network of fine fibrils rather than thicker banded fibrils as seen by electron microscopy of vertebrate interstitial matrices. In contrast to vertebrate collagens that require pepsin digestion for solubilization, large polymeric structures of Hcol-I can be isolated

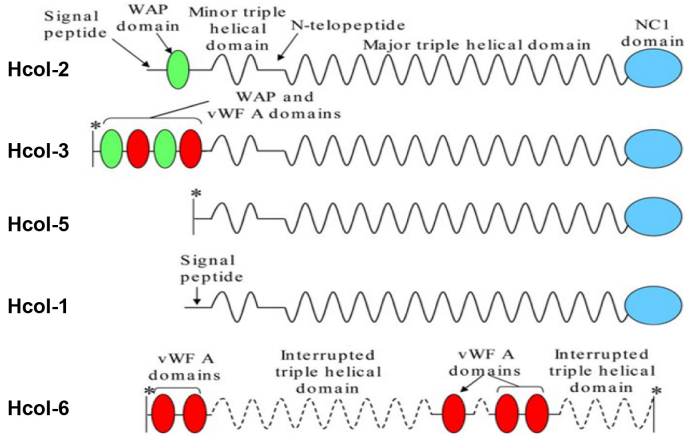

Fig. 3. Summary of the proposed structure of each of the *Hydra* fibrillar collagens. *The complete cDNA sequences are known for Fibrillar collagens Hcol-1, -2, -3 and -5 and the deduced structures are based on rigorous biochemical structural analysis. (Zhang* et al.*, 2007; modified with permission of the author).*

from the ECM of *Hydra* under native conditions.

Several factors are responsible for the special structure of *Hydra* Hcol-I. These factors include: 1) a low content of proline in the triple helical domain that is only about 40% that of vertebrate collagens: 2) a reduced degree of inter-chain cross-linking due to the lack of classical consensus sequences for lysine/lysine-aldehyde derived covalent bonds; and 3) most importantly, altered post-translational processing that results in retention of the N-terminal propeptide-like domain in the mature molecule. Combined, these factors result in a more flexible fibrillar collagen that can bend on itself as suggested by the early ultrastructural studies of Davis and Haynes (1968).

2) Hydra Fibrillar Collagens: Hcol2, Hcol3, Hcol5, Hcol6. Next to be identified through molecular cloning techniques were four fibrillar collagens named, Hcol2, Hcol3, Hcol5 and Hcol6 (Zhang et. al, 2007). Hcol6 was only a partial sequence of a collagen gene with a unique structural organization consisting of multiple von Willebrand factor-A domains interspersed with interrupted collagenous triple helices. Hcol2 and Hcol5 have major collagenous domains of classical length (1020 amino acid residues), whereas the equivalent domain in Hcol3 is shorter (969 residues). The N-propeptide of Hcol2 contains a whey-acid-protein four-cysteine repeat (WAP) domain, and the equivalent domain of Hcol3 contains two WAP and two von Willebrand factor-A domains (Fig. 3).

Phylogenetic analyses revealed that the *Hydra* fibrillar collagen genes form a distinct clade that appeared related to the protostome/deuterostome A-clade of fibrillar collagens. Data base searches revealed that Hcol2, Hcol5, and Hcol6 are highly conserved, which also provided preliminary evidence for the expression of a B-clade fibrillar collagen. *In situ* hybridization indicated an ectoderm expression pattern along the entire longitudinal axis of *Hydra* for the Hcol2 and Hcol3 fibrillar collagens as was previously reported for Hcol1 (Deutzmann,.et al., 2000). *Hydra* Hcol2 and Hcol3 also have high expression in the tentacles and forming buds. Fibrillar collagen Hcol5 has high expression in the tentacles, foot process, and forming buds of adult polyps while Hcol6 expression is high at the base of the tentacles and in forming buds. During head regeneration it is expressed in the ectoderm as observed with Hcol1. Hcol6 is expressed to a much lower degree than the other fibrillar collagens and its expression pattern is restricted to the base of the tentacles and to forming buds (Zhang *et al*., 2007).

3) Non-ECM collagens of Hydra. To be complete, it should be pointed out that *Hydra* has a variety of what are called “mini collagens”. These collagens are localized within the capsule of nematocysts and are not associated with *Hydra*’s ECM. They have a unique and interesting structure due to their role in maintaining high hydrostatic pressures within the cavity of the nematocyst capsule. Because these collagens are not associated with *Hydra*’s ECM, they will not be further discussed in this review, but the reader is referred to articles pertinent to their unique structure and function (Kurz *et al*., 1991; Holstein *et al*., 1994), see also in this issue the review by Beckmann and Ozbek (2012).

Evidence for other types of matrix components in Hydra ECM

During functional studies using a pharmacological approach, evidence was obtained by Sarras *et al.* (1991, 1993) and Zhang and Sarras (1994) indicating that proteoglycans also exist in the *Hydra* ECM. These experiments involved analysis of the effect of molecules that block proteoglycan biosynthesis during head regeneration (Sarras *et al*., 1991), *Hydra* cell aggregate formation, and during cell migration as studied in *Hydra* grafting experiments (Zhang and Sarras, 1994). The validity of these pharmacological studies was confirmed using pulse-chase autoradiographic techniques to demonstrate that proteoglycan-associated molecules such as SO4 were in fact blocked from appearing in the ECM following treatment with such agents as β-xyloside as compared to its inactive isomer, α-xyloside. These studies indicated that any blockage in proteoglycan biosynthesis resulted in a blockage in *Hydra* morphogenesis (head regeneration and cell aggregate morphogenesis) and a retardation in normal i-cell migration, suggesting that proteoglycans are components of the *Hydra* ECM. As of this date however, proteoglycans have not been isolated from *Hydra* ECM and therefore this evidence is indirect in nature.

The structural relationship of Hydra ECM molecular components to its flexible biophysical properties

Hydra has enhanced the flexible nature of its fibrillar collagens such as Hcol-I by reducing its proline content and by decreasing the degree of lysine/lysine inter-chain cross-links (Deutzmann, 2000; Zhang *et al*., 2007). In addition, alterations in the structure of *Hydra* collagen type IV are also observed and these structural alterations likely lead to a more flexible Hcol-IV network following polymerization of the ECM. In the case of Hcol-IV, C-terminal interactions and lateral aggregation occur too, but as indicated previously, a stable 7S domain is not formed. The reason might be that formation of a highly ordered 7S domain would impose steric constraints so that the four molecules leave at preferred angles. This might favor the formation of rigid basement membranes as needed by vertebrates to contribute to the shape of organs; but for formation of the flexible ECM of *Hydra*, as discussed above, a more relaxed cross-linking involving some cysteine residues in the N-terminal region might be more advantageous. In any case, the results for *Hydra* clearly show that the formation of a 7S domain is not essential for formation of a collagen type IV network. This is also supported by earlier findings for the worm *Ascaris suum* (Noelken *et al*., 1986). Taken together, the structural properties of *Hydra* Hcol-IV and the organism’s fibrillar collagens, such as Hcol-I, contribute to the flexible nature of *Hydra* ECM.

In summary, whereas vertebrates need a tight extracellular matrix that can withstand high tensile forces such as in the Achilles tendon, *Hydra* needs a highly flexible ECM, so that the animal can continuously contract and extend. The molecular and biochemical studies of *Hydra* Collagen Type IV and *Hydra* Fibrillar Collagens, such as Hcol-I, were required to understand the flexible nature of *Hydra* ECM.

Biogenesis of *Hydra* ECM is intimately tied to regeneration in this simple metazoan

As will be further discussed in the next section, *Hydra* ECM is required for general morphogenesis, pattern formation, and cell differentiation to occur in the adult polyp. This has been determined through head and foot regeneration studies and through experiments utilizing *Hydra* cell aggregates. Development of *Hydra* cell aggregates involves complete morphogenesis of the *Hydra* body structure within 48-72 hours from a cell pellet formed

from non-enzymatically dissociated cells obtained from the adult polyp (Gierer *et al.*., 1972; Bode, 1974, Sarras *et al.*, 1993). In all processes studied (head regeneration, foot regeneration, and development of *Hydra* cell aggregates), formation of a new ECM must occur for morphogenesis to proceed. If ECM biogenesis is blocked or perturbed in any way, morphogenesis is stopped. This has been shown through a broad array of approaches to include: 1) use of pharmacological agents (Sarras *et al.*, 1991), 2) blocking antibodies to *Hydra* ECM components (Sarras *et al.*, 1993), 3) fragments of ECM components that are used to compete in the normal polymerization of the matrix components (Zhang *et al.*, 1994), and 4) anti-sense RNA to ECM components introduced through electrophoresis techniques (Shimizu *et al.*, 2002).

The sequence of events associated with *Hydra* ECM biogenesis indicates that cross-talk occurs between the ectoderm and endoderm and through signals emanating from the ECM to both epithelial layers. A general description of *Hydra* ECM biogenesis is depicted in Fig. 4. The need for ECM biogenesis during experimentally-induced head or foot regeneration is explained by the unique elastic properties of *Hydra*'s ECM. Removal of the head or foot or a simple incision along the gastric tube wall of *Hydra* results in a retraction of the ECM from the cut edge, thereby creating a newly sealed epithelial bilayer that has no ECM between the two cell layers (Shimizu *et al.*, 2002). This ECM-deficient bilayer changes its morphology so that cells become flattened compared to the more cuboidal to columnar cellular phenotype normally seen along *Hydra*'s body wall (Fig. 4). Soon after sealing (within 1 hr post-decapitation), this bilayer immediately begins to synthesize a new ECM. The process involves initial up-regulation of ECM component genes within 3 hrs of decapitation (in the case of head regeneration experiments as shown in Fig. 4). In this process, basal lamina mRNA expression (e.g. laminin and Hcol-IV) is specific to endodermal cells while interstitial matrix components expression (e.g. Hcol1, 2, and 3) is specific to the ectoderm layer of cells. Within 7 hrs of decapitation, basal lamina (BL) proteins are secreted from the endoderm and a newly formed BL is seen associated with the basal extracellular border of both the ectoderm and endoderm. Through ECM receptor systems (Agbas and Sarras, 1994) BL components trigger synthesis of fibrillar collagens, such as Hcol1, that are secreted from the ectoderm and polymerize in the interstitial matrix zone between to the two BL layers (Shimizu *et al.*, 2002). The linkage between BL formation and interstitial matrix component translation and secretion is supported by RNA antisense experiments in which laminin translation is blocked and subsequent Hcol1 translation and secretion from the ectoderm is prevented (Shimizu *et al.*, 2002), thereby blocking ECM formation and perturbing morphogenesis of the bilayer to a head or foot structure. Because *Hydra* cell aggregates form from dissociated tissues, the initial epithelial bilayer that forms lacks an ECM. If one uses pharmacological agents, blocking antibodies to *Hydra* ECM components, or fragments of ECM components to perturb ECM polymerization in *Hydra* cell aggregate; morphogenesis of the aggregates beyond the cystic stage will not occur (Zhang *et al.*, 1994).

Taken in total, these studies indicate that 1) ECM biogenesis is essential for *Hydra* morphogenesis and 2) ECM biogenesis is a complicated process involving cross-talk between the endoderm and ectoderm as well as signaling from the ECM to the cells of the bilayer. This cross-talk involves sequential synthesis of basal

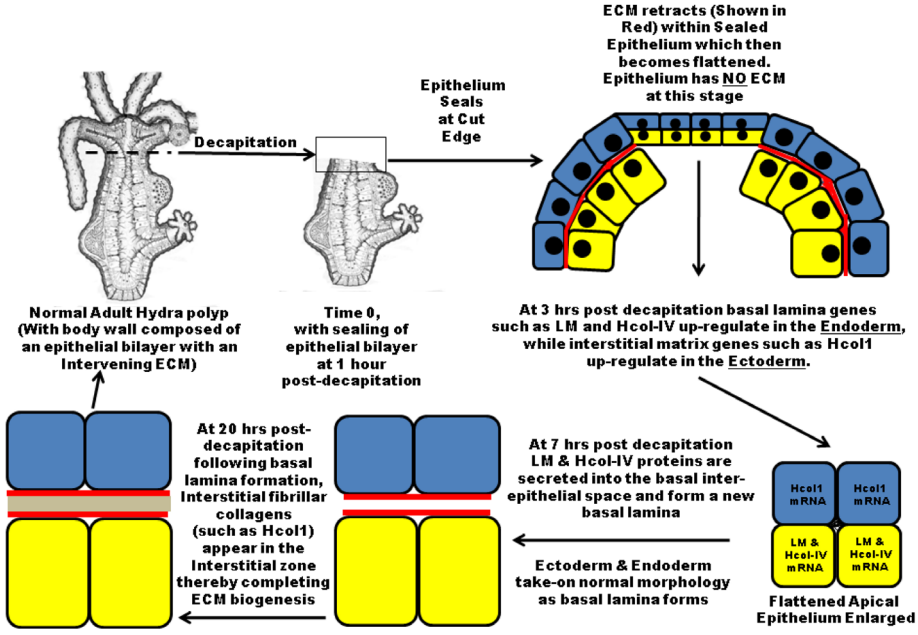

Fig. 4. Extracellular matrix (ECM) biogenesis following decapitation of *Hydra*. *Experimentally-induced regeneration in* Hydra *is typified by surgical removal of the head pole in the adult* Hydra *polyp. This is a simple procedure involving excision of the head pole with a small scalpel. When decapitation is initiated, the open head pole (the gastric cavity is now open to the aqueous environment) seals within 1 hour by fusion of the two cut ends of the apical epithelial bilayer (Shimizu et al., 2002). The elastic properties of* Hydra *ECM cause the entire matrix to retract at the cut ends resulting in the sealed epithelial bilayer to now lack an intervening ECM. Because of the lack of an ECM, the apically sealed epithelial bilayer alters its morphology and becomes flattened as compared to the normal cuboidal-columnar phenotype that body wall cells display. Within 3 hours, an up-regulation of ECM genes is observed in the ectoderm and endoderm (Shimizu et al., 2002). This up-regulation could be due to the abrupt lack of an ECM and subsequent ectoderm/endoderm cell to cell signaling. By 7 hours, basal lamina components such as laminin and collagen type IV (Hcol-IV) are translated and secreted into the basal inter-epithelial compartment where the ECM is normally located. The basal lamina (BL) polymerizes at the basal border of both the endoderm and ectoderm. Once this occurs, the epithelial bilayer begins to resume its normal phenotype. Following the appearance of the BL, and 20 hours after decapitation was initiated, interstitial fibrillar collagens such as Hcol1 begin to be translated and appear between the two previously formed basal lamina layers adjacent to the basal extracellular border of the ectoderm and endoderm (Shimizu et al., 2002). This likely involves signaling events between the basal lamina components and the ectodermal cell layer from which Hcol1 is produced. These signals between the basal lamina components and basally located ECM receptor plasma membrane complexes (e.g. integrins) trigger initiation of ECM-specific cellular pathways that are required for biogenesis of the ECM. Once the interstitial matrix polymerizes, the ECM is now completely polymerized and the normal adult Hydra body wall structure is reformed. This permits pattern formation processes to complete head regeneration morphogenesis.*

lamina (endoderm) and interstitial matrix (ectoderm) components that will in turn signal appropriately timed translation and secretion of ECM proteins resulting in the polymerization of a well ordered matrix. As with higher invertebrates, this also involves ECM receptor complexes that trigger appropriate signaling pathways associated with ECM biogenesis processes.

The relationship of ECM structure to cell-ECM interactions in *Hydra*

Cell-ECM interactions are central to normal tissue homeostasis, a broad spectrum of disease states, as well as developmental processes (Matejas et.al. 2010; Kim *et al..*, 2011; Watt and Fujiwara, 2011; Wicksgtrom *et al..*, 2011; Yurchenco, 2011). While this review has focused on the relationship of *Hydra* ECM to the overall structural and cell behavior characteristics of this invertebrate, it should be noted that cell-ECM interactions have been determined to be important to a number of developmental processes in *Hydra* beyond general morphogenesis. These processes include, 1) cell proliferation, 2) cell migration, 3) cell differentiation, and as previously discussed, 4) morphogenesis. These cell-ECM interactions in *Hydra* are listed in Table 2 with those references from which the studies were reported. A role for the ECM in the modulation of cell proliferation in *Hydra* was first reported by Zhang *et al.,* (1974). These studies showed that introduction of exogenous matrix protein domains during ECM formation in *Hydra* resulted in alterations in cell proliferation rates. Likewise, alteration of ECM structure or exogenous introduction of blocking antibodies to matrix components or matrix component domains would retard i-cell migration in *Hydra* grafting experiments (Zhang and Sarras, 1994). *In vitro* studies with isolated nematocytes have also indicated dependence of cell-ECM interactions for cell migratory processes in *Hydra* (Gonzalez *et al.*, 1991; Ziegler and Stidwill, 1992; Stidwill and Christen, 1998). As discussed above, a number of studies involving head regeneration (Sarras *et al.*, 1991) and morphogenesis of *Hydra* cell aggregates (Sarras *et al.*, 1993) have clearly shown that any perturbation of ECM formation will result in blockage of *Hydra* morphogenesis. In addition, studies involving *Hydra* cell aggregates (Zhang *et al.*, 1994) or *Hydra* foot regeneration (Leontovich *et al.*, 2000) have shown that perturbation of ECM structure or turnover can affect cell differentiation in the adult *Hydra* polyp. Signaling motifs in these cell/ECM interactions can be exposed or cryptic.

In this regard, the action of *Hydra* matrix metalloproteinases has been shown to be involved in exposing potentially cryptic signaling sites within ECM components (Leontovich *et al.*, 2000). *Hydra* matrix metalloproteinase (HMMP) has been shown to specifically affect the maintenance of the phenotype of basal disk cells through mechanisms that remain unclear (Leontovich *et al.*, 2000). A similar observation has been reported for *Hydra* metalloproteinase-1 that is localized to the tentacle ECM and has been shown to be involved in the maintenance of tentacle battery cell phenotypic markers (Yan *et al.*, 1995, 2000). As reviewed by Schmid *et al.,*1999), the importance of cell-ECM interactions during morphogenesis and cell differentiation extends beyond *Hydra* to a number of classes within Cnidaria. Finally, and most recently, a new *in vivo* labelling technique for *Hydra* collagen 1 and laminin was used to track the fate of ECM in all body regions of the animal (Aufschnaiter *et al.*, 2011). These studies revealed that *Hydra* "tissue movements" are largely displacements of epithelial cells together with associated ECM (Aufschnaiter *et al.*, 2011). In contrast, during the evagination of buds and tentacles, extensive movement of epithelial cells relative to the matrix was observed together with local ECM remodelling (Aufschnaiter *et al.*, 2011). Taken in total, these studies show a clear role for cell-ECM interactions in *Hydra*. These interactions are a dynamic process that is intimately associated with the continuous turnover of epithelial cells along the body axis of *Hydra*.

In conclusion, it is important to note, that *Hydra* Cell/ECM interactions represent highly conserved processes within the animal kingdom given the early divergence of *Hydra* during metazoan evolution. Accordingly, the functional role of *Hydra* ECM in a variety of its cellular and developmental processes is seen replicated in more complicated vertebrate systems (Kim *et al..*, 2011; Kruegel and Miosge, 2010; Watt and Fujiwara, 2011; Wicksgtrom *et al..*, 2011; Yurchenco, 2011). This serves to reinforce the fundamental nature of these cell-ECM processes and highlights their importance to the cell biology of all metazoans. This also reminds us that although *Hydra* is considered a relatively "simple" organism, this does not mean that its cellular and molecular biology is anything but simple to understand. In this regard, future studies with *Hydra* should be directed at 1) understanding how the epithelial bilayer of *Hydra* coordinates the formation of its ECM through cell signaling pathways and 2) the precise mechanisms underlying the role of ECM in *Hydra* morphogenesis and cell differentiation.

TABLE 2

DEVELOPMENTAL PROCESSES IN *HYDRA* THAT HAVE BEEN REPORTED TO INVOLVE CELL-ECM INTERACTIONS

1. Porosity of *Hydra* ECM as it facilitates Cell/Cell communication between the Ectoderm and Endoderm.	Shimizu et al., 2008
2. *Hydra* ECM as it relates to cell turnover and the budding process.	Aufschnaiter et. al., 2011
3. Cell proliferation during morphogenesis as monitored in *Hydra* cell aggregates.	Zhang et al., 1994
4. Cell migration under in vivo conditions in grafting experiment and under in vitro conditions with isolated *Hydra* nematocytes using ECM coated culture plates.	Gonzalez-Agosti and Stidwill, 1991; Ziegler and Stidwill, 1992; Zhang and Sarras, 1994; Stidwill and Christen, 1998
5. Cell differentiation/transdifferentiation of basal disk cells and battery cells and cell differentiation in Hydra cell aggregates.	Zhang et al., 1994; Yan et al., 1995; Yan et al., 2000; Leontovich et al., 2000
6. Epithelial morphogenesis of the head or foot as monitored in regeneration experiments and in the adult polyp as monitored in Hydra cell aggregate experiments.	Barzansky and Lenhoff, 1974; Sarras et al., 1991; 1993; 1994; Yan et al.,1995; Yan et al., 2000; Deutzmann et al., 2000; Fowler et al., 2000

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