The Company of Biologists

METHODS & TECHNIQUES

Establishing and maintaining primary cell cultures derived from the ctenophore *Mnemiopsis leidyi*

Lauren E. Vandepas[1,*], Kaitlyn J. Warren[2,*], Chris T. Amemiya[1,3] and William E. Browne[4,‡]

ABSTRACT

We have developed an efficient method for the preparation and maintenance of primary cell cultures isolated from adult *Mnemiopsis leidyi*, a lobate ctenophore. Our primary cell cultures are derived from tissue explants or enzymatically dissociated cells, and maintained in a complex undefined ctenophore mesogleal serum. These methods can be used to isolate, maintain and visually monitor ctenophore cells to assess proliferation, cellular morphology and cell differentiation in future studies. Exemplar cell types that can be easily isolated from primary cultures include proliferative ectodermal and endodermal cells, motile amebocyte-like cells, and giant smooth muscle cells that exhibit inducible contractile properties. We have also derived 'tissue envelopes' containing sections of endodermal canal surrounded by mesoglea and ectoderm that can be used to monitor targeted cell types in an *in vivo* context. Access to efficient and reliably generated primary cell cultures will facilitate the analysis of ctenophore development, physiology and morphology from a cell biological perspective.

KEY WORDS: Cell biology, Marine invertebrate, Endoderm, Ectoderm, Tissue culture

INTRODUCTION

In vitro primary cell cultures are typically derived from tissue explants, are often composed of a variety of cell types that prove useful for monitoring a diverse range of cellular phenomena, and are regularly used as a proxy for the *in vivo* cellular environment (Nargeot and Simmers, 2011; Yina et al., 2016). Unlike immortalized monotypic cell lines that have been selected or genetically manipulated to proliferate indefinitely, primary cell cultures generally have a limited lifespan. Marine invertebrate primary cell cultures have been used to assess disease in important aquaculture taxa (e.g. crustaceans and bivalves), explore diverse and/or novel cell types, and understand symbioses (Cai and Zhang, 2014; Davy et al., 2012; Huete-Stauffer et al., 2015; Rinkevich, 2005; Weis et al., 2008). Despite their obvious value in unraveling complex cellular phenomena, *in vitro* cell cultures remain an underutilized experimental resource across most metazoans (reviewed in Rinkevich, 2005).

Ctenophora is a phylum of gelatinous marine invertebrates named for their rows of unique comb-like cilia (Eschscholtz, 1829).

[1]Department of Biology, University of Washington, Seattle, WA 98195, USA.
[2]Department of Biology, University of Miami, Coral Gables, FL 33146, USA.
[3]Benaroya Research Institute at Virginia Mason, Seattle, WA 98101, USA.
[4]Department of Biology, University of Miami, Coral Gables, FL 33146, USA.
*These authors contributed equally to this work

‡Author for correspondence (wbrowne@bio.miami.edu)

W.E.B., 0000-0001-8200-6489

Ctenophores have two readily recognizable germ layers, an outer ectoderm and an inner endoderm, separated by a jelly-like layer of mesoglea primarily composed of water and extracellular matrix (ECM) material. This mesogleal space contains muscle, nerve, mesenchymal and amebocyte-like cells (Hernandez-Nicaise, 1991). The outer ectoderm covers the exterior of the body, lines the inner surface of the pharynx, and consists of a range of cell types including ciliary cells associated with the comb rows and apical organ, as well as an extensive ectodermal nerve net. The inner endoderm layer contributes to organs associated with the branched gastrovascular through-gut (Hyman, 1940; Presnell et al., 2016).

Despite the absence of a distinct mesodermal germ layer, ctenophore-specific giant smooth muscle cells (Dayraud et al., 2012; Hernandez-Nicaise and Amsellem, 1980; Hernandez-Nicaise et al., 1984) are distributed throughout the mesoglea, and striated muscle cells have been documented in one species (Mackie et al., 1988). The ctenophore nervous system is composed of a subepithelial nerve net, mesogleal nerves and tentacular nerves (Jager et al., 2011; Moroz et al., 2014; Norekian and Moroz, 2016). Recent genome sequencing and analyses suggest that early animal evolution may have involved major losses and/or gains of canonical nerve and muscle cell types (Moroz et al., 2014; Ryan, 2014; Ryan et al., 2013).

The unique attributes of ctenophores are, in part, due to the long separation of the ctenophore lineage from other metazoans (Dunn et al., 2008; Whelan et al., 2015). Although many unique cell types are present in ctenophores, their basic cell biology has been understudied, contributing to the misrepresentation of ctenophores as 'simple' organisms. The availability of an efficient and reliable primary cell culturing system will play an important role in understanding the phylum's unique evolutionary history and cell biology. Currently, no methodologies for producing and maintaining ctenophore primary cell culture systems have been published. Our goal was to develop an easy and reliable method for generating primary cell cultures that can be utilized to isolate representative cell types from the lobate ctenophore *Mnemiopsis leidyi* A. Agassiz 1865. The protocols presented here will aid in the *in vitro* isolation and characterization of distinct cell types associated with this enigmatic group of metazoans.

MATERIALS AND METHODS
Target tissue dissection and preparation
Animals in good health are best for generating robust representative primary cell cultures. Selected animals were washed with 0.2 µm filter-sterilized artificial seawater (FSW; Instant Ocean, Instant Ocean Spectrum Brands, Blacksburg, VA, USA) to remove debris and mucus. For specific cell types of interest, the appropriate tissue type or organ can be targeted for isolation. In these experiments, both lobes of an individual *M. leidyi* were excised and rinsed three times in FSW with 1% penicillin+streptomycin (pen/strep) solution (Sigma Aldrich, St Louis, MO, USA). FSW pen/strep solution was

prepared by diluting a 100× pen/strep stock solution at 10,000 U ml^{-1} penicillin and 10,000 µg ml^{-1} streptomycin to 1×.

Ctenophore mesoglea serum preparation

One excised lobe was sliced into pieces, divided among several microcentrifuge tubes and homogenized using a pestle. The ctenophore mesoglea serum (CMS) homogenate was then spun for 30 min at maximum speed at room temperature to sediment debris. The resulting supernatant was mixed 1:1 with 0.2 µm-filtered seawater+pen/strep solution. Centrifugation was repeated if debris remained in solution. Alternatively, 100 µl of 0.5 mm stainless steel beads (Next Advance, Averill Park, NY, USA) were added and tubes were placed in a Bullet Blender homogenizer (Next Advance) set to high for 5 min at 4°C or until the homogenate could be smoothly pipetted. CMS homogenate was subsequently heat inactivated at 56°C for 30 min on low rotation to reduce viscosity, lyse remaining cells and eliminate heat-intolerant microbes. To control for potential immune responses, serum was prepared from the same individual from which cells were isolated. CMS can be stored for 72 h at 4°C. ECM material may continue to sediment during cold storage and thus was centrifuged briefly to aggregate precipitates prior to use.

Tissue explant preparation

The remaining lobe was wounded with a shallow cut along the surface using a scalpel and allowed to recover for 25 min for recruitment of cells to the wound site. Small pieces of tissue were cored out of the lobe using a microcentrifuge tube with the bottom sheared off. This produces jagged explant edges that inhibit rapid healing. Individual tissue explants were placed on glass microscope slides fitted with 0.5 mm-deep silicone isolators and covered with 300 µl of CMS at room temperature. The best results were obtained by incubating the tissue explants and subsequent primary cell cultures at 16°C. Plastic cell culture dishes were also used, with no significant changes in cell viability. Explant remnants were removed at 48 h post-isolation to prevent overcrowding.

Preparing dissociated cells

Chambered glass slides (ThermoFisher Scientific, Waltham, MA, USA) were coated with a collagenous matrix (Matrigel, Corning Incorporated, Tewksbury, MA, USA) at a 1:15 dilution in FSW+1% pen/strep to promote cell attachment. Excised tissue was placed in 5 ml FSW+pen/strep in a glass tissue grinder and gently homogenized with a loose-fitting pestle (Wheaton, Millville, NJ, USA). The resulting homogenate was centrifuged for 10 min at 350 g at room temperature to pellet cells. To dissociate the cell pellets, 10 ml of 0.25% trypsin/EDTA (in FSW+pen/strep) (ThermoFisher Scientific, Waltham, MA, USA) solution was added, and the tube was rotated at ~75 rpm for 10–15 min at room temperature. Dissociated cells were repelleted by centrifuging for 10 min at 350 g at room temperature. Residual trypsin was inactivated by resuspension in CMS medium (FSW+1% pen/strep+10% CMS) followed by centrifugation for 10 min at 350 g at room temperature and subsequent resuspension in media for plating. Primary cell cultures tolerate a wide range of final CMS percentages.
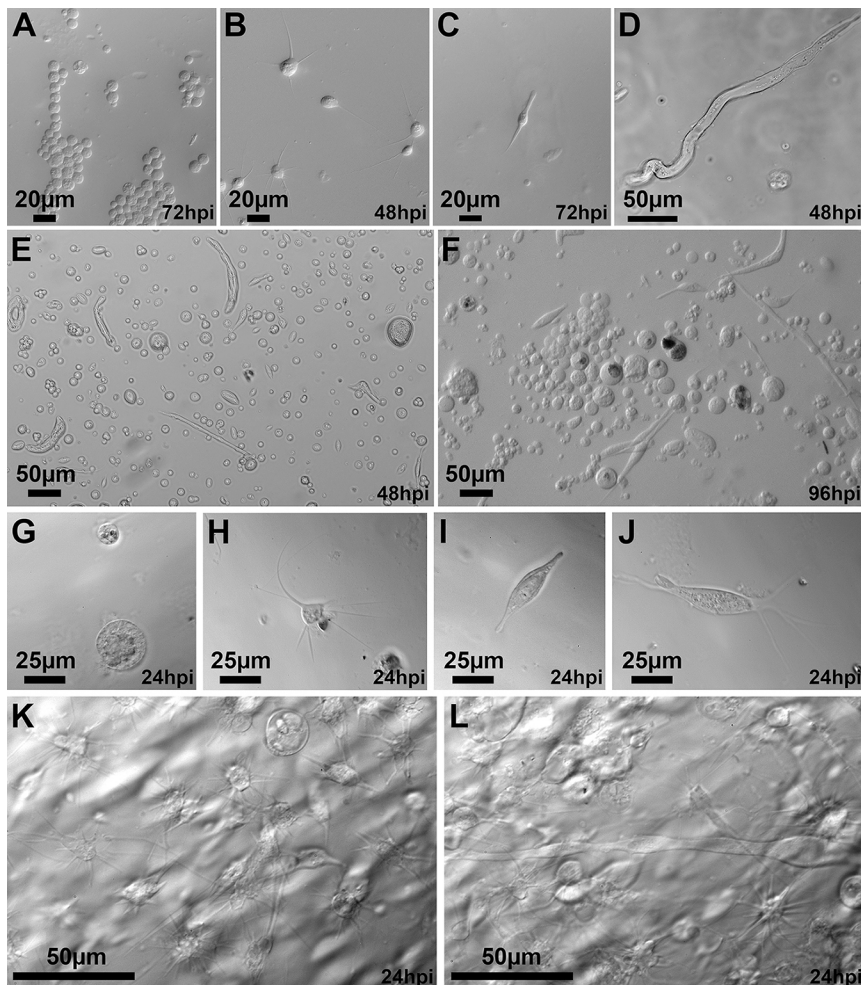
Fig. 1. Exemplar cell types isolated from *Mnemiopsis leidyi* primary cell culture. (A) A 72 h post-isolation (hpi) culture of ectodermally derived cells dividing in culture, producing linear chains. (B) Motile cells at 48 hpi displaying long processes (see also Movie 1). (C) A bipolar cell at 72 hpi displaying long processes. (D) A giant smooth muscle cell at 48 hpi. (E) A wide-field view of diverse isolated cell types from tissue explant at 48 hpi. (F) Pigmented vacuolated cells are among the cell types isolated from tissue explant containing endoderm (96 hpi). (G–J) Diverse unknown cell types at 24 hpi from trypsin enzymatic isolation, with processes displaying a variety of morphologies. (K,L) Examples of incomplete enzymatic isolation resulting in thin layers of loosely associated cells, allowing for localized cell–cell interactions to be observed *in situ*.

Increased percentages of CMS generally yield a higher-viscosity medium. Our enzymatically dissociated cell cultures were maintained in 10% CMS media, similar to serum concentrations in traditional mammalian cell culture media.

Primary cell culture maintenance

Slides containing cells and explants were kept in humidified chambers at 14–16°C for best results, or at room temperature. Approximately 24 h after seeding, slides were screened for the presence of desired cell types. Remaining explant tissue aggregates were removed at ~48 h to prevent overcrowding and CMS media were refreshed at ~72 h intervals by removing 50% of the existing volume and replacing it with fresh CMS. Regular CMS exchanges improve cell culture viability by replenishing nutrients, reducing metabolic waste products and removing suspended cell debris. Cell cultures with CMS media exchanges could be maintained from several days to several weeks.

Tissue envelope preparation

Tissue envelopes can be used in conjunction with cell cultures to study cells of interest in an *in vivo* context. A small (3×5 mm) section of target tissue was excised using a scalpel. Tissue sections were then placed on a chambered slide, covered with CMS media and incubated at 14–16°C. Typically, tissue envelopes sealed the wound site within 4–6 h and remained intact for 72–120 h.

Vital dye loading

Exemplar fluorescent vital dyes were added at appropriate final concentrations: MitoTracker (Molecular Probes), 250 nmol l^{-1}; LysoTracker (Molecular Probes), 33.33 nmol l^{-1}; Hoechst 33342 (Molecular Probes), 1.25 µg ml^{-1}. Vital dyes were introduced into primary cell cultures at 72 h post-seeding in CMS media brought to room temperature. To expose primary cells to vital dyes, a 50% exchange of vital dye-spiked CMS media was added and incubated for 45 min at 14–16°C. Cells were washed with three gentle 50% exchanges of fresh CMS. To successfully access deep layers in tissue envelopes, a narrow gauge needle was used to introduce vital dye solutions into endodermal canal luminal space.

RESULTS AND DISCUSSION

Like other primary cell culture systems, ctenophore primary cells have a finite lifespan under culture conditions. Explants from different locations and/or tissues yield a range of tissue-specific cell types. Using tissue explants alone will not recapitulate all cell types in culture. Explant derived cultures have an enriched representation of cells that readily slough from the edges or are competent to migrate out of the explant. Using trypsinization for cell isolation may yield a wider variety of representative cell types. We sought to characterize the cell types present in our cultures using visual assays, assessment of cell motility and contractile properties. We also performed selected morphological cell type counts during the initial establishment and early maintenance of explant-derived primary cell cultures (Fig. S1). Several distinct cell morphologies were consistently observed at 72 h: hyper-elongated, bipolar, multipolar and round cells (Fig. 1).

We tested contractile properties of individual cells by mechanical stimulation using a glass needle. If a cell exhibited a direct response to stimulation, it was considered to have contractile properties. In general, medial mechanical stimulation with a glass needle did not elicit a contractile response from any of the cell types observed, including giant smooth muscle cells, whereas stimulation of extreme ends and prominent processes typically elicited dramatic

contractile responses (Fig. 2, compare left and right panels). Primary cell cultures were also exposed to MitoTracker, LysoTracker and Hoechst to detect the presence of mitochondria, lysosomes and nuclei, respectively (Fig. 3).

Ectodermally derived round cells were the most abundant. As assessed by cell counts, these cells proliferate in culture at the rate of ~1 division per 24 h, typically in clonally related chains (Fig. 1A). Our analyses suggest they are non-motile and possess a high nuclear:cytoplasmic volume ratio. Many of the bipolar and multipolar cell morphologies observed use highly dynamic processes for motility (Fig. 1B; Movie 1). Some of these cell types also demonstrated contractile properties. Both bipolar and multipolar cell morphologies, also termed spindle shaped or stellate, are comparable to those found previously (Dodson, 2010).

Our protocol facilitates the isolation of giant smooth muscle cells (Fig. S2A). These cells are generally hyper-elongated and multinucleated (Figs 2E,F, 3A,B) and range from ~300 to 1000 µm in length (Fig. 1D; Fig. S2). Mechanical stimulation with a needle at either end of these cells typically initiates a dramatic contraction (Fig. 2A–D). After ~72 h in culture, isolated giant smooth muscle cells regularly develop increasingly chaotic processes along their entire length (Fig. S2C). This atypical branched morphology is not usually observed among giant smooth muscle cells *in vivo*.

Primary cell cultures derived from tissue explants containing endodermal canals include digestive cells that, *in vivo*, asymmetrically line the inner endodermal canal wall (Presnell et al., 2016). These large digestive cells can be identified based on the presence of large pigmented organelles (Figs 1F, 3E). LysoTracker localizes to these organelles, suggesting that they are
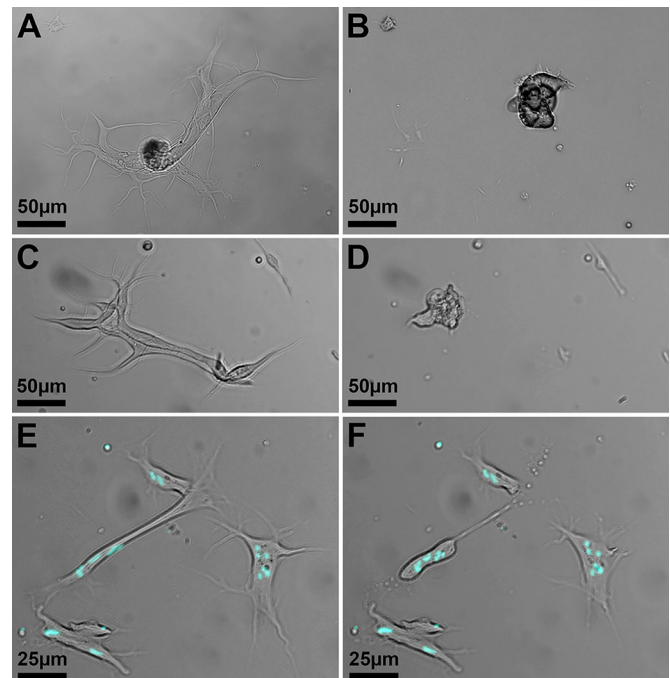
Fig. 2. Multinucleated presumptive muscle cells with long processes and contractile properties. (A) Undisturbed cell in a relaxed state. (B) The same cell shown in A, after stimulation at a distal process with a glass needle tip. (C) Undisturbed cell in a relaxed state. (D) The same cell shown in C after stimulation of a distal process with a glass needle tip. (E) Hoechst staining of presumptive multinucleated muscle cells bearing long processes. (F) The central cell exhibits a contractile response to short wavelength blue light stimulation (compare with E).
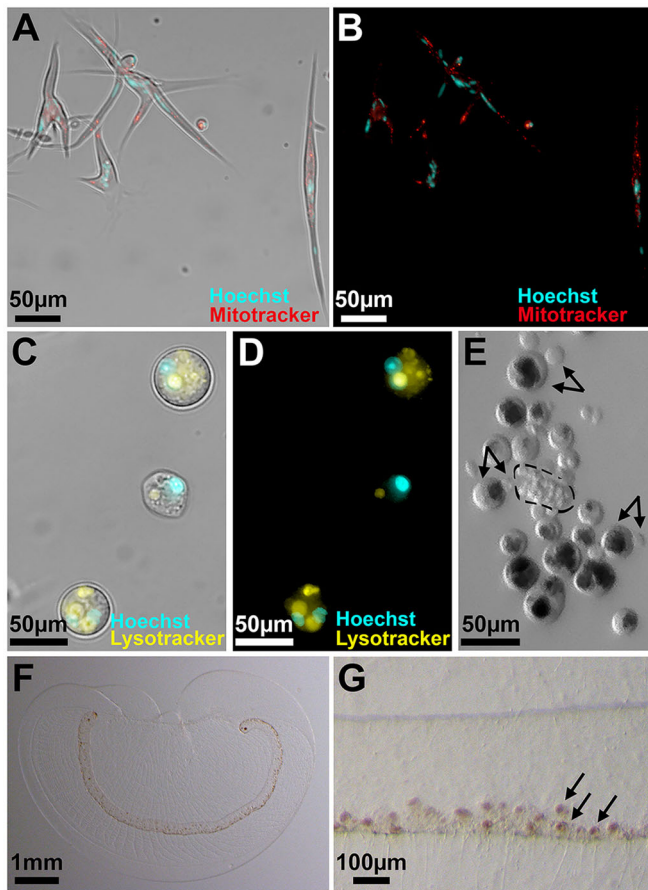
Fig. 3. Vital dye staining of cultured *Mnemiopsis* cells. Nuclei are labeled blue with Hoechst, mitochondria are labeled red with Mitotracker, and acidic lysosomal vacuoles are labeled yellow with Lysotracker. (A) Merged differential interference contrast (DIC) and fluorescence image showing a high concentration of mitochondria in multinucleated presumptive muscle cells. (B) Fluorescence-only image of cells shown in A. (C) Merged DIC and fluorescence image of binucleated endodermal cells containing large lysosomal vacuoles. (D) Fluorescence-only image of the cells shown in C. (E) Subcultured endoderm-derived cells. Dashed outline marks the central proliferative mass occupied by relatively small cells; larger differentiating cells are on the periphery of the central mass. Arrows mark pairs of cells resulting from asymmetric cleavage in which one daughter cell remains relatively small and one daughter cell has differentiated as a larger cell containing pigmented lysosomes. (F) Brightfield image of prepared tissue envelope with an endodermal canal segment containing pigmented cells (see also Movie 3). (G) Brightfield image of endodermal canal section in a tissue envelope. Arrows show the *in vivo* asymmetric localization of the pigmented cells in C–E.

acidic vacuoles (Fig. 3C,D). These ciliated cells are motile and typically binucleated, suggesting they are terminally differentiated. However, our cell count data indicated that these cells appeared to both persist and increase in number over time (Fig. S1). Further observation revealed a class of small proliferative, presumably pluripotent progenitor cells present in our primary cultures that are competent to differentiate via asymmetric cleavage into mature digestive cells (Fig. 3E).

In ~30% of our explant-derived primary cell cultures, mature swimming sperm cells appeared 72–144 h post-isolation (Fig. S3, Movie 2). Interestingly, this was also observed in cultures derived from explants containing only ectoderm and mesoglea. We suspect cell types competent to differentiate into sperm cells may not be restricted to a particular germ layer. Intriguingly, in *Mnemiopsis* a homolog of the sex determination *doublesex* gene, *DmrtA*, is both

alternatively spliced and expressed in a wide ectodermal domain during development (Reitzel et al., 2016).

To more closely visualize interactions between cell types of interest, we also developed *in vitro* tissue envelopes. We focused on the morphologically distinct endodermally derived digestive cells. These tissue envelopes contain small sections of endodermal canal (Fig. 3F). For example, the large digestive cells that are commonly isolated in culture from explants containing endoderm line the inner wall facing the lumen of the endodermal canals in these tissue envelopes (Fig. 3G). These large digestive cells regularly slough from the inner wall into the canal lumen (Movie 3), suggesting a high turn-over rate for this terminally differentiated cell type. Based on the intersection of observations from both primary cell culture and tissue envelopes, we are confident that the primary physiological function of these readily isolatable endodermal cells is closely allied with absorption of digested material from the gut lumen (Presnell et al., 2016).

These are the first reported ctenophore primary cell culture protocols specifically developed to aid in the investigation of unique aspects of cellular differentiation and physiology associated with ctenophore development from a cell biological perspective (Presnell et al., 2016). Marine invertebrates display a wide range of life histories and many have remarkable regenerative capabilities. Stem cell research in marine invertebrates is being explored in a number of lineages (Rinkevich, 2011). Given the ability of ctenophores to readily regenerate (Martindale, 2016), the application of cell culture techniques to better understand the cellular basis of this regeneration is likely to have significant experimental value.

We have validated this technique with other species of ctenophores, including *Pleurobrachia bachei* and *Bolinopsis infundibulum*. Primary cell cultures derived from ctenophores will not only facilitate the characterization of cell types unique to ctenophores but also importantly contribute a broader understanding of the evolution of animal cell type diversity.

Supplementary information
Supplementary information available online at
http://jeb.biologists.org/lookup/doi/10.1242/jeb.152371.supplemental

References
Cai, X. and Zhang, Y. (2014). Marine invertebrate cell culture: a decade of development. *J. Oceanogr.* **70**, 405-414.
Davy, S. K., Allemand, D. and Weis, V. M. (2012). Cell biology of cnidarian-dinoflagellate symbiosis. *Microbiol. Mol. Biol. Rev.* **76**, 229-261.
Dayraud, C., Alié, A., Jager, M., Chang, P., Le Guyader, H., Manuel, M. and Quéinnec, E. (2012). Independent specialisation of myosin II paralogues in

muscle vs. non-muscle functions during early animal evolution: a ctenophore perspective. *BMC Evol. Biol.* **12**, 107.

Dodson, M. B. (2010). Rapid wound repair in the Ctenophore *Mnemiopsis* spp. Master's Thesis, Auburn University, USA.

Dunn, C. W., Hejnol, A., Matus, D. Q., Pang, K., Browne, W. E., Smith, S. A., Seaver, E., Rouse, G. W., Obst, M., Edgecombe, G. D. et al. (2008). Broad phylogenomic sampling improves resolution of the animal tree of life. *Nature* **452**, 745-749.

Eschscholtz, F. (1829). *System der Acalyphen: Eine ausführliche Beschreibung aller medusenartigen Strahltiere*. Berlin: VDM Verlag.

Hernandez-Nicaise, M.-L. (1991). Ctenophora. In *Microcopic Anatomy of the Invertebrates*, Vol. 2 (ed. F. W. Harrison), pp. 359-418. New York: Wiley-Liss.

Hernandez-Nicaise, M. L. and Amsellem, J. (1980). Ultrastructure of the giant smooth muscle fiber of the ctenophore *Beroe ovata*. *J. Ultrastruct. Res.* **72**, 151-168.

Hernandez-Nicaise, M.-L., Nicaise, G. and Malaval, L. (1984). Giant smooth muscle fibers of the ctenophore *Mnemiopsis leidyi*: ultrastructural study of *in situ* and isolated cells. *Biol. Bull.* **167**, 210-228.

Huete-Stauffer, C., Valisano, L., Gaino, E., Vezzulli, L. and Cerrano, C. (2015). Development of long-term primary cell aggregates from Mediterranean octocorals. *In Vitro Cell. Dev. Biol. Anim.* **51**, 815-826.

Hyman, L. H. (1940). *Protozoa through Ctenophora*. New York: McGraw-Hill.

Jager, M., Chiori, R., Alié, A., Dayraud, C., Quéinnec, E. and Manuel, M. (2011). New insights on ctenophore neural anatomy: immunofluorescence study in Pleurobrachia pileus (Müller, 1776). *J. Exp. Zool B Mol. Dev. Evol.* **316B**, 171-187.

Mackie, G. O., Mills, C. E. and Singla, C. L. and **Zoomorphology 107.** (1988). Structure and function of the prehensile tentilla of *Euplokamis* (Ctenophora, Cydippida). *Zoomorphology* **107**, 319-337.

Martindale, M. Q. (2016). The onset of regenerative properties in ctenophores. *Curr. Opin. Genet. Dev.* **40**, 113-119.

Moroz, L. L., Kocot, K. M., Citarella, M. R., Dosung, S., Norekian, T. P., Povolotskaya, I. S., Grigorenko, A. P., Dailey, C., Berezikov, E., Buckley, K. M. et al. (2014). The ctenophore genome and the evolutionary origins of neural systems. *Nature* **510**, 109-114.

Nargeot, R. and Simmers, J. (2011). Neural mechanisms of operant conditioning and learning-induced behavioral plasticity in Aplysia. *Cell. Mol. Life Sci.* **68**, 803-816.

Norekian, T. P. and Moroz, L. L. (2016). Development of neuromuscular organization in the ctenophore Pleurobrachia bachei. *J. Comp. Neurol.* **524**, 136-151.

Presnell, J. S., Vandepas, L. E., Warren, K. J., Swalla, B. J., Amemiya, C. T. and Browne, W. E. (2016). The presence of a functionally tripartite through-gut in ctenophora has implications for metazoan character trait evolution. *Curr. Biol.* **26**, 2814-2820.

Reitzel, A. M., Pang, K. and Martindale, M. Q. (2016). Developmental expression of "germline"- and "sex determination"-related genes in the ctenophore *Mnemiopsis leidyi*. *Evodevo* **7**, 17.

Rinkevich, B. (2005). Marine invertebrate cell cultures: new millennium trends. *Mar. Biotechnol.* **7**, 429-439.

Rinkevich, B. (2011). Cell cultures from marine invertebrates: new insights for capturing endless stemness. *Mar. Biotechnol.* **13**, 345-354.

Ryan, J. F. (2014). Did the ctenophore nervous system evolve independently? *Zoology* **117**, 225-226.

Ryan, J. F., Pang, K., Schnitzler, C. E., Nguyen, A.-D., Moreland, R. T., Simmons, D. K., Koch, B. J., Francis, W. R., Havlak, P., Program, N. C. S. et al. (2013). The genome of the ctenophore Mnemiopsis leidyi and its implications for cell type evolution. *Science* **342**, 1242592.

Weis, V. M., Davy, S. K., Hoegh-Guldberg, O., Rodriguez-Lanetty, M. and Pringle, J. R. (2008). Cell biology in model systems as the key to understanding corals. *Trends Ecol. Evol.* **23**, 369-376.

Whelan, N. V., Kocot, K. M., Moroz, L. L. and Halanych, K. M. (2015). Error, signal, and the placement of Ctenophora sister to all other animals. *Proc. Natl. Acad. Sci. USA* **112**, 5773-5778.

Yina, S., Chenghua, L., Weiwei, Z., Zhenhui, W. and Zhimeng, L. (2016). The first description of complete invertebrate arginine metabolism pathways implies dose-dependent pathogen regulation in *Apostichopus japonicus*. *Sci. Rep.* **6**, 23783.