

The tunicate *Ciona*: a model system for understanding the relationship between regeneration and aging

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The use of the tunicate *Ciona intestinalis* as a model system to study the relationship between regeneration and aging is reviewed. *Ciona* has powerful regeneration capacities, which fade with age. Some additional benefits are a relatively short life span, the ability to study regeneration *in vitro*, the close phylogenetic relationship between tunicates and vertebrates, and the host of molecular tools already established in this system. The neural complex (NC), the oral siphon (OS), and the oral siphon pigment organs (OPO) have high capacities for regeneration. However, these organs show an inverse relationship between rate of regeneration and age. The ability to regenerate a complete OS disappears in the oldest animals of a natural population, probably due to the inability to form a blastema at the wound site. Effects on blastema formation could also be involved in the reduction of NC regeneration capacity. The fidelity of OPO restoration is also compromised by excess differentiation of precursor cells in local siphon niches in the oldest animals. The *Ciona* model provides a pathway to understand the molecular basis of these phenomena.

Keywords: *Ciona intestinalis*; neural complex; oral siphon; oral siphon sensory organs

Introduction

Regeneration is one of biology's major unsolved problems. Although some vertebrates have strong regenerative capacities, the ability to accurately replace lost parts often decreases with maturity. For example, amputated appendages are capable of replacement in anuran tadpoles, but this capacity is lost after metamorphosis (Girvan et al. 2002), and the slowly developing hind limb, but not the more rapidly developing fore limb, of opossums is capable of regeneration (Mizell 1968). Even in humans, which have no limb regeneration capacities, young children are sometimes able to replace the tips of severed fingers, but this ability is reduced as they mature (Illingworth & Barker 1980). Therefore, a central problem in the field of regenerative biology comes into sharp focus: explaining the fading or loss of regenerative potential during development and aging.

Model systems in which the effects of aging on tissue repair and regeneration can be addressed in the same species are not generally available. The long life span and complexity of vertebrates limit their effectiveness in studying the relationship between aging and regeneration. The invertebrate model systems used to study aging, such as *Drosophila melanogaster* and *Caenorhabditis elegans*, are also insufficient to address this problem since their regenerative abilities are minimal. In contrast, the tunicate *Ciona intestinalis* (Figure 1), a solitary ascidian, has powerful regeneration capacities,

a relatively short life span, and a reduced capacity to accurately replace missing tissues with age (Dahlberg et al. 2009; Auger et al. 2010).

Tunicates are chiefly known for mosaic embryonic development (Satoh 1994). However, this rigid form of development is dramatically reversed, presumably during metamorphosis, when the capacity is attained to replace missing body parts following injury. This article describes *Ciona* as a model system for studying the decline and eventual loss of regenerative capacity during aging.

The *Ciona* model system

Ciona has many desirable attributes that make it an excellent model for studies of regeneration and aging. First, there is a wealth of information on *Ciona* development, particularly about the embryonic phase (Satoh 1994). For example, embryonic cell lineages are well known and could be explored to reveal the embryonic cells that produce the multipotent cells involved in adult regeneration. Second, the *Ciona* genome has been sequenced and annotated (Dehal et al. 2002), potentially permitting numerous genes to be assayed for their involvement in regeneration. Third, transgenic animals are available that express molecular markers in specific tissues (Sasakura 2007) and these markers can be used to track their participation in regenerative phenomena. A transgenic line with GRF

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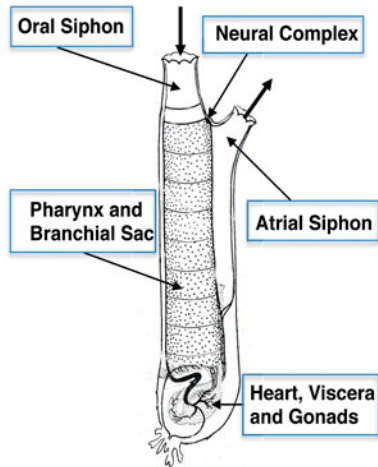


Figure 1. *Ciona intestinalis*. Thick arrows show the directions of water currents into and out of the siphons. Note: Modified from Millar (1953).

expressed throughout the adult nervous system has already provided a key marker in *Ciona* regenerative biology (Dahlberg et al. 2009; Auger et al. 2010). Fourth, *Ciona* can be grown from egg to adult in closed marine aquaria (Joly et al. 2007), allowing precise determination of the age of individual animals. Fifth, *Ciona* adult organs can be cultured as explants (Moss et al. 1998; Auger et al. 2010), allowing regenerative events to be manipulated *in vitro*. Finally, the tunicates have been inferred as the closest living relatives of vertebrates (Bourlat et al. 2006; Delsuc et al. 2006), suggesting that the mechanisms of regenerative decay discovered in *Ciona* may be relevant to those of vertebrates.

Ciona life history

The *Ciona* life cycle has embryonic, larval, juvenile, and adult phases (Satoh 1994). Beginning at the two-cell stage, every blastomere has a unique fate. Removal of a blastomere results in the development of an incomplete larva, cultured blastomeres, or partial embryos form only the derivatives expected from the fate map, and bisected larvae cannot regenerate the other part. After dispersal by swimming, larvae settle head first on a substrate and undergo metamorphosis, which begins with the retraction of the larval tail into the trunk. The adult body plan is organized in post-metamorphic juveniles.

The *Ciona* life span is relatively short in nature or the laboratory: larvae metamorphose a few days after fertilization, juvenile development takes about 10 days, and the young adults become reproductive, grow isometrically, and die in 12–18 months (Berrill 1947; Millar 1952; Dybern 1965; Petersen et al. 1995). In animals living in the same microhabitats, there is a positive

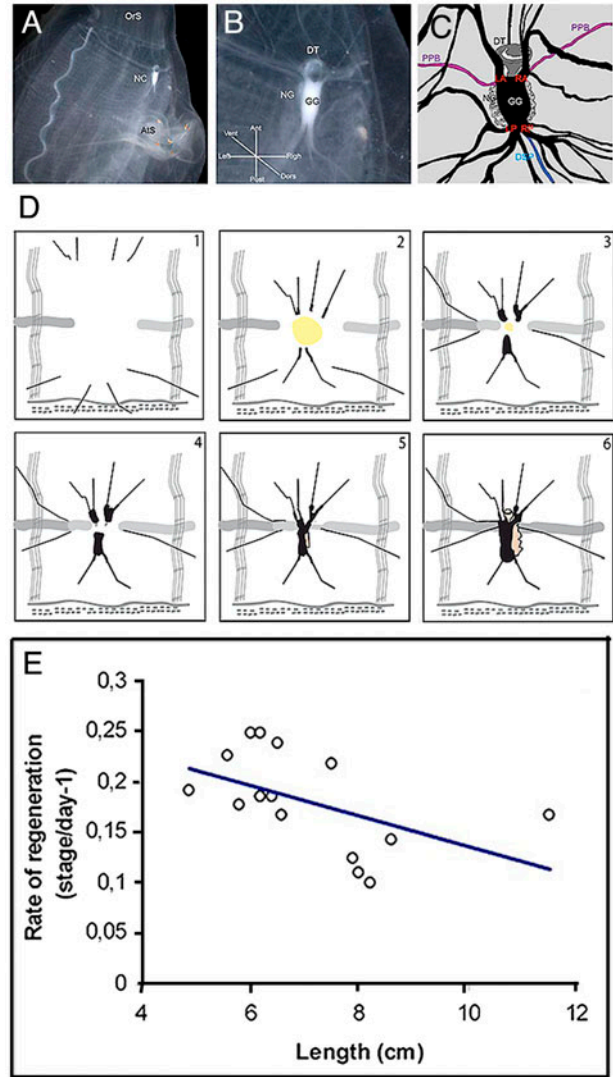


Figure 2. Neural complex structure and regeneration. Top row: (A–C) the NC. OrS: oral siphon. AtS: atrial siphon. GG: cerebral ganglion. NG: neural gland. DSP: dorsal strand plexus. DT: ciliated funnel. LA and RA: left and right anterior nerves. LP and AP: left and right posterior nerves. PPB: peripharyngeal band. Middle row: drawings 1–5 show progressive stages of NC regeneration. Bottom row: rate of NC regeneration is inversely related to animal length.

Note: Modified from Dahlberg et al. (2009).

correlation between age and body length, and thus the latter can be used as a proxy for age (Berrill 1947; Millar 1953; Jeffery 2012).

A short history of *Ciona* regeneration research

Research on *Ciona* regeneration began around the turn of the twentieth century. Schultze (1899) ablated the neural complex (NC), the organ unit including the cerebral ganglion (brain), neural gland, and associated

ciliated structures (Figures 1 and 2(A–C)), and found that it was replaced within about a month. Hirschler (1914) separated parts of the adult body with their component organs and recorded their capacity to regenerate a complete animal. When an animal was split into a basal portion containing mostly viscera and surrounding organs and a distal portion containing the branchial sac, pharynx, and siphons (see Figure 1), the basal portion was capable of regenerating a distal portion – if it contained a piece of the branchial sac – but the distal portion could not regenerate the basal portion. When pieces of the body were separated at positions more distal to the visceral-pharyngeal border, and eventually only the siphons, the basal parts always regenerated the distal parts but not vice versa. Subsequently, regeneration of the NC (Bollner et al. 1992, 1993, 1995, 1997) and siphons (Fox 1924; Sutton 1953; Whittaker 1975) were confirmed by many investigators, and are the focus of current studies to understand the relationship between regeneration and aging.

NC regeneration and aging

The hub of the NC is the brain, which contains the cell bodies of neurons extending into the siphons, body wall, and viscera (Figure 2(A–C)). The neural gland is connected to the pharynx via the ciliated funnel on its anterior side and it tapers on its posterior side into the dorsal strand (Figure 2(C)). The dorsal strand contains a plexus of neurons and putative neuroblasts with immunoreactivity for gonadotropin-releasing hormone (GnRH) (Bollner et al. 1997). The brain also contains GnRH positive cells, as well as neurons immunoreactive for substance P, neurokinin A (NKA), cholecystokinin–gastrin, insulin, and GABA (Bollner et al. 1992, 1995, 1997). Most of the neurons marked by these hormones and neurotransmitters reappear during regeneration in locations similar to those present before NC ablation (Bollner et al. 1992, 1995, 1997). Surprisingly, however, NKA and insulin immunoreactive cells were not detected in the regenerated brain (Bollner et al. 1992), suggesting that NC regeneration process is incomplete at the cellular level.

NC regeneration can also be monitored by behavioral responses (Dahlberg et al. 2009). The ascidian brain controls several body reflexes (Mackie et al. 2006): (1) whole body contraction and (2) contractions restricted to one or both siphons, which are elicited by touching a siphon rim, and (3) ‘cross siphon’ contraction that is produced by stimulating tentacles in the oral siphon (OS). These responses are lost after NC ablation but gradually regained as connections reappear between the regenerating NC and the severed neural networks. They offer another set of markers that will be useful in assaying the physiological process of brain regeneration, but have yet to be tested in old versus young animals.

Live imaging, transgenic animals, and cell-labeling methods have added further tools for analysis of brain regeneration (Dahlberg et al. 2009). NC regeneration has been divided into six successive stages by imaging methods (see Figure 2(D)): (1) healing of the wound, (2) further healing and merger of the severed nerve stumps at the anterior and posterior edges of the ablation site, (3) swelling, enlarging, and fusing of the nerve stumps at their tips, (4) reappearance of the brain and neural gland, (5) enlargement of the NC and reconnection of the anterior and posterior nerves, and (6) restoration of a morphologically complete, albeit smaller, NC. These results revealed that regenerative activities on all sides of the wound contribute to the formation of a new NC.

Fedele (1938) proposed that neuroblasts migrating from the dorsal strand plexus into the wound site are responsible for brain regeneration. This idea was supported by the initial re-appearance of GnRH immunoreactive cells at the posterior end of the regenerating brain, near the dorsal strand plexus (Bollner et al. 1997). The GnRH immunoreactive cells failed to incorporate BrdU into DNA, suggesting that they were born prior to the onset of regeneration or that they trans-differentiate from other cell types (Bollner et al. 1995). The possibility of trans-differentiation in the absence of cell proliferation would be consistent with regenerating a smaller NC. Once visualization of NC regenerative events was improved using transgenic animals (Dahlberg et al. 2009), GFP-labeled neural cells were seen to contribute to the brain on all sides of the regenerate, not only the side closest to the dorsal strand. Additionally, GFP and EdU labeling were seen at the tip of each cluster of severed neurons extending into the wound site, suggesting the presence of a small blastema of proliferating cells. Thus, current data support a local source of cells involved in NC regeneration, although additional roles of long-distance migration from the dorsal strand or other parts of the body and trans-differentiation cannot be excluded.

An inverse relationship exists between animal length and the rate of NC regeneration (Dahlberg et al. 2009). Since size is related to age in wild *Ciona*, this correlation supports the idea of a decline in NC regeneration capacity with age. This might also be an explanation for the absence of insulin and NKA immunoreactive cells in the regenerated brain (Bollner et al. 1992) because larger animals might have been selected for NC ablation. Age-related changes in large animals might also explain the surprising lack of NC regeneration, even a year after ablation, in other tunicates (Hisaw et al. 1966; Mackie et al. 2006), although species differences are also a possibility. The potential relationship between the fidelity of NC regeneration and age warrants further study in laboratory-reared animals.

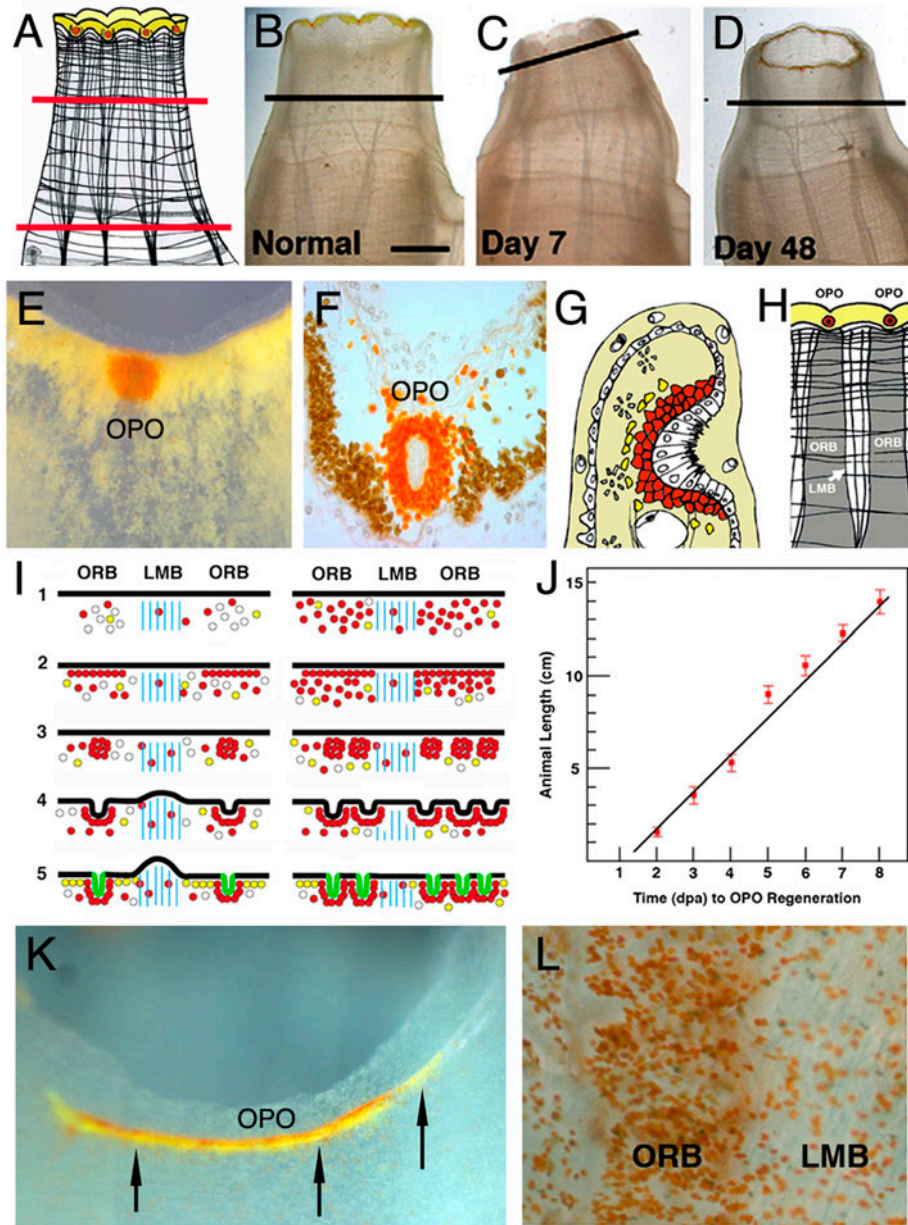


Figure 3. OS structure and regeneration. (A and B) Diagram (A) and image of a living animal (B) showing the OS. Red lines in A indicate the location of amputation planes. (C and D) Images of the OS of living animals at two stages of regeneration. (E and F) Image of an OPO in a living animal (E) and in histological section (F). Diagrams of an OS rim in section showing the ciliated epidermal pit surrounded by a cup of orange pigment cells (G) and a part of the OS (H) wall with OPO in register with the underlying oral siphon regeneration band (ORB). LMB: longitudinal muscle band. (I) Diagram showing the sequence of OPO regeneration (1–5) in young (left) and old (right) animals (see Jeffery 2012 for details). (J) The negative relationship between the rate of OPO regeneration and animal length. Dpa: days post amputation. (K) OPO (arrows) regeneration in an old animal. (L) Siphon explant from an old animal showing excessive differentiation of orange pigment cells in the ORB.

Note: Modified from Millar (1953) for (A and B), from Auger et al. (2010) for (B–H, and J), from Jeffery (2012) for (I, K, and L).

OS regeneration and aging

The OS is the contractile opening of the pharynx and the feeding conduit in *Ciona* adults (Figures 1 and 3(A and B)). It contains a ring of tentacles at its base and a ring of pigmented sensory organs (sometimes called ocelli)

near its rim (Dilly & Wolken 1973). Most individuals contain eight sensory oral pigment organs (OPO), although a minority shows seven or nine OPO (Millar 1953; Auger et al. 2010). Each OPO consists of a pit-like crypt of ciliated epidermal cells surrounded by a

cup of orange pigment cells (Figure 3(E–G)). The OPO are positioned within notches between the siphon lobes, where they punctuate an otherwise continuous band of yellow pigment encircling the siphon rim (Figure 3(A, B, E, and F)). The OS wall contains diffuse circular muscle fibers and longitudinal muscle bands (LMB). The LMB alternate with regions mostly free of muscle fibers called OPO regeneration bands (ORB), which are in register with the OPO above them (Figure 3(H)), and the likely source of cells involved in their replacement.

After removal of the distal portion of an OS (Figure 3(A), top red line), OPO were replaced with fidelity in both structure and number (Figure 3(C); Auger et al. 2010). The replacement process involves (1) the alignment of newly differentiated orange pigment cells at the edge of the wound, (2) the condensation of these orange pigment cells into spots, and (3) the formation of orange pigment cups around new epidermal crypts (Figure 3(I), left). Although replacement of the entire OS requires about a month, the OPO and other distal structures are reformed in eight days or less, before the underlying proximal structures appear. Precise fidelity of OPO replacement also occurs following several repetitive cycles of OS amputation and regrowth, indicating that the patterning mechanisms involved in regeneration are highly accurate. In contrast, OS amputation at its base (Figure 3(A), bottom red line) caused the regeneration of up to 16 OPO after a single ablation and regrowth cycle, showing that the normally robust regeneration process can be compromised.

Hirschler (1914) suggested that ablated siphons were replaced by migration of cells from distant source(s) in the body. However, Auger et al. (2010) demonstrated that a blastema is involved in siphon regeneration, suggesting a local source of precursor cells. The possible roles of local and long distance sources were further investigated in two ways (Auger et al. 2010). First, the distal margins of the OS were UV irradiated prior to amputation, and this blocked OPO regeneration. Second, amputated OS stumps were excised from the body and cultured *in vitro*, and these explants regenerated normal OPO at their distal margins. Thus, it was concluded that the source of OPO progenitor cells is local and that they are localized in the ORB before the onset of regeneration (Figure 3(H)). Further studies will be necessary to identify the sources of other cell types in the regenerating OS.

Auger et al. (2010) also demonstrated a strong negative correlation between the rate of OPO regeneration and length: under identical conditions, the smallest (and youngest) animals in a wild population regenerated OPO in only two days, whereas the largest (and oldest) required about eight days to reform the ring of OPO (Figure 3(J)). It was also found that old animals lacked the ability to form a blastema in the OS stump, and this was proposed as a probable cause of the effects on

regeneration (Jeffery 2012). Old animals also showed defects in OPO regeneration (Figure 3(I), right): they formed supernumerary and/or malformed OPO (Figure 3(K)) and were unable to reproduce to produce normal OPO following multiple amputation cycles (Jeffery 2012). Finally, explant cultures of OS stumps from old animals did not form OPO, but instead differentiated excessive numbers of orange-pigmented cells in the ORB niche (Jeffery 2012). These results suggest that the capacity for OS regeneration is compromised by multiple defects during aging.

Conclusions and prospectus

The tunicate *Ciona* is an excellent model to study the relationship between regeneration and aging. During youth, it shows powerful and robust regeneration but this capacity diminishes during aging, and is lost in the oldest animals. *Ciona* appears to have three different phases in its life cycle with regard to regeneration: (1) the embryonic and larval stages, in which regeneration is absent, (2) the juvenile and young adult stages, in which regeneration is robust, and (3) old age, in which regeneration is gradually reduced and may be completely lost. A major cause of the loss of regeneration in old animals may be their inability to develop a blastema of proliferating cells at the wound site. Further progress in understanding the mechanisms of *Ciona* regeneration will require identifying the cells and molecules involved in generating this blastema and how they are modified during aging. The future prospects for understanding the molecular basis of decreased regeneration capacity during aging is promising due to the superb molecular tool kit that has been established in *Ciona*.

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References

- Auger H, Sasakura Y, Joly J-S, Jeffery WR. 2010. Regeneration of oral siphon pigment organs in the ascidian *Ciona intestinalis*. *Developmental Biology*. 339:374–389.
- Berrill NJ. 1947. The development and growth of *Ciona*. *Journal of the Marine Biological Association of the United Kingdom*. 26:616–625.
- Bollner T, Beesley PW, Thorndyke M. 1992. Pattern of substance P- and cholecystokinin-like immunoreactivity during regeneration of the neural complex in the ascidian *Ciona intestinalis*. *The Journal of Comparative Neurology*. 325:572–580.
- Bollner T, Beesley PW, Thorndyke MC. 1993. Distribution of GABA-like immunoreactivity during post-metamorphic development and regeneration of the nervous system in the ascidian *Ciona intestinalis*. *Cell and Tissue Research*. 272:553–561.

- Bollner T, Beesley PW, Thorndyke MC. 1997. Investigation of the contribution from peripheral GnRH-like immunoreactive 'neuroblasts' to the regenerating central nervous system in the protochordate *Ciona intestinalis*. Proceedings of the Royal Society B: Biological Sciences. 264:1117–1123.
- Bollner T, Howalt S, Thorndyke MC, Beesley PW. 1995. Regeneration and post-metamorphic development of the central nervous system in the protochordate *Ciona intestinalis*: a study with monoclonal antibodies. Cell and Tissue Research. 279:421–432.
- Bourlat SJ, Juliusdottir T, Lowe CJ, Freeman R, Aronowicz J, Kirschner M, Lander ES, Thorndyke M, Nakano M, et al. 2006. Deuterostome phylogeny reveals monophyletic chordates and the new phylum Xenoturbellida. Nature. 444:85–88.
- Dahlberg C, Auger H, Dupont S, Sasakura Y, Thorndyke M, Joly J-S. 2009. Refining the model system of central nervous system regeneration in *Ciona intestinalis*. PLoS One. 4(2):e4458.
- Dehal P, Satou Y, Campbell RK, Chapman J, Degnan B, De Tomaso A, Davidson B, Di Gregorio A, Gelpke M, Goodstein M, et al. 2002. The draft genome of *Ciona intestinalis*: insights into chordate and vertebrate origins. Science. 298:2157–2167.
- Delsuc F, Brinkmann H, Chourrout D, Philippe H. 2006. Tunicates and not cephalochordates are the closest living relatives of vertebrates. Nature. 439:965–968.
- Dilly PN, Wolken JJ. 1973. Studies on the receptors of *Ciona intestinalis*: IV. The ocellus in the adult. Micron. 4:11–29.
- Dybern BI. 1965. The life cycle of *Ciona intestinalis* (L) f. typical; in relation to environmental temperature. Oikos. 16:109–131.
- Fedele M. 1938. Il sistema nervoso degli 'Ascidiacea' nel piano di organizzazione dell' Cordati [The nervous system of Ascidiacea in the chordate organization plan]. Atti dell' Accademia Nazionale Lincei Rendiconti, Series. 6(27):370–376.
- Fox HM. 1924. Note on Kammerer's experiments with *Ciona* concerning the inheritance of an acquired character. Journal of Genetics. 14:89–91.
- Girvan JE, Olson WN, Hall BK. 2002. Hind-limb regeneration in the dwarf African clawed frog, *Hymenochirus boettgeri* (Anura: Pipidae). Journal of Herpetology. 36:537–543.
- Hirschler J. 1914. Über die Restitutions- und Involutionen-vorgänge bei operierten Exemplaren von *Ciona intestinalis* Flem [Concerning the restitution and involution processes in *Ciona intestinalis* Flem. operated individuals]. Archiv für Mikroskopische Anatomie. 85:205–227.
- Hisaw FL Jr, Botticelli CR, Hisaw FL. 1966. A study of the relation of the neural gland-ganglionic complex to gonadal development in an ascidian, *Chelyosoma productum* Simpson. General and Comparative Endocrinology. 7:1–9.
- Illingworth CM, Barker AT. 1980. Measurement of electrical currents emerging during the regeneration of amputated finger tips in children. Clinical Physics and Physiological Measurement. 1:87–89.
- Jeffery WR. 2012. Siphon regeneration capacity is compromised during aging in the ascidian *Ciona intestinalis*. Mechanisms of Ageing and Development. 133:629–636.
- Joly J-S, Kano S, Matsuoka T, Auger H, Hirayama K, Satoh N, Awazu S, Legendre L, Sasakura Y. 2007. Culture of *Ciona intestinalis* in closed systems. Developmental Dynamics. 236:1832–1840.
- Mackie GO, Burighel P, Caicci F, Manni L. 2006. Innervation of ascidian siphons and their responses to stimulation. Canadian Journal of Zoology. 84:1146–1162.
- Millar RH. 1952. The annual growth and reproductive cycle in four ascidians. Journal of the Marine Biological Association of the United Kingdom. 31:41–61.
- Millar RH. 1953. *Ciona*. In: Colman JS, editor. LMBC memoirs on typical British marine plants and animals. Liverpool (UK): Liverpool University; p. 1–123.
- Mizell M. 1968. Limb regeneration: induction in the newborn opossum. Science. 161:283–286.
- Moss C, Beesley PW, Thorndyke MC, Bollner T. 1998. Preliminary observations on ascidian and echinoderm neurons and neural explants *in vitro*. Tissue and Cell. 30:517–524.
- Petersen JK, Schou O, Thor P. 1995. Growth and energetics in the ascidian *Ciona intestinalis*. Marine Ecology Progress Series. 120:175–184.
- Sasakura Y. 2007. Germline transgenesis and insertional mutagenesis in the ascidian *Ciona intestinalis*. Developmental Dynamics. 236:1758–1767.
- Satoh N. 1994. Developmental biology of ascidians. Cambridge (UK): Cambridge University Press.
- Schultze LS. 1899. Die Regeneration des Ganglions von *Ciona intestinalis* L [The regeneration of the ganglion in *Ciona intestinalis* L]. Jena Z f Naturwiss. 33:263–344.
- Sutton MF. 1953. The regeneration of the siphons of *Ciona intestinalis* L. Journal of the Marine Biological Association of the United Kingdom. 32:249–286.
- Whittaker JR. 1975. Siphon regeneration in *Ciona*. Nature. 255:224–225.