ELSEVIER

Tissue and Cell 38 (2006) 193–201

Tissue&Cell

www.elsevier.com/locate/tice

Programmed cell death in vegetative development: Apoptosis during the colonial life cycle of the ascidian *Botryllus schlosseri*

S. Tiozzo [1], L. Ballarin *, P. Burighel, G. Zaniolo

University of Padova, Department of Biology, via U. Bassi 58/B, 35121 Padova, Italy

Received 7 December 2005; received in revised form 17 February 2006; accepted 24 February 2006
Available online 24 April 2006

Abstract

Programmed cell death (PCD) by apoptosis is a physiological mechanism by which cells are eliminated during embryonic and post-embryonic stages of animal life cycle.

During asexual reproduction, the zooids of colonial ascidians originate from an assorted cell population instead of a single zygote, so that we assume that regulation of the equilibrium among proliferation, differentiation and cell death may follow different pathways in comparison to the embryonic development. Here we investigate the presence of apoptotic events throughout the blastogenetic life cycle of the colonial ascidian *Botryllus schlosseri*, by means of terminal deoxynucleotidyl transferase dUTP Nick End Labeling (TUNEL) coupled with histochemical and electron microscopy techniques. The occurrence of low levels of morphogenetic cell death suggests that, in contrast to what happens during sexual development (embryogenesis and metamorphosis), apoptosis does not play a pivotal role during asexual propagation in botryllid ascidian. Nevertheless, PCD emerges as a key force to regulate homeostasis in adult zooids and to shape and modulate the growth of the whole colony.

Keywords: *Botryllus*; Ascidians; Apoptosis; Blastogenesis; Colonial life cycle

1. Introduction

Cell proliferation and programmed cell death (PCD) are two basic mechanisms which take part and cooperate in modelling and defining body architecture of developing metazoans. In particular PCD occurs during both organ development and the regulation of tissue homeostasis (Zahir and Weaver, 2004). The most widespread type of PCD is apoptosis, which leads to characteristic cell phenotypes that allows the clearance of effete cells by phagocytes. With its fine control on chromatin remodelling, cell cycle regulation, cytoskeleton organisation and plasma membrane composition, apoptosis represents one of the main innovation of eukaryotes, so that its molecular machinery is well conserved since primitive metazoans (Aravind et al., 1999; Cikala et al., 1999; Koonin and Aravind, 2002). Besides its role in regulating the plasticity of developing organisms (Namba et al., 1997), apoptosis takes part also in the elimination of transient or senescent organs and structures in response to physiological events or suboptimal environmental conditions (Gordon, 1977).

Considering the importance of apoptosis in embryonic development and adult life of animals, it is of interest to investigate the role of the process in the course of blastogenesis. For this kind of approach colonial ascidians represent useful model organisms for both the variety of asexual types of propagation and their peculiar phylogenetic position of invertebrate chordates. In tunicates, apoptosis has been studied during the embryogenesis and metamorphosis of few solitary ascidians (Chambon et al., 2002; Jeffery, 2002a,b; Bates, 2004; Cole and Meinertzhagen, 2004; Tarallo and Sordino, 2004), and during the takeover phase of the colonial species *Botryllus schlosseri* (Burighel and Schiavinato, 1984; Lauzon et al., 1992, 1993, 2002; Cima et al., 2003).

* Corresponding author. Tel.: +39 049 8276197; fax: +39 049 8276199.
E-mail address: loriano.ballarin@unipd.it (L. Ballarin).
[1] Present address: Department of Pathology, Hopkins Marine Station of Stanford University, Pacific Grove, California.

0040-8166/$ – see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tice.2006.02.003

B. schlosseri is a cosmopolitan, clonally modular ascidian which inhabits shallow waters and harbours throughout the world (Kott, 1989). Each colony arises from a tadpole larva that, after a short swimming phase, settles to a substratum and undergoes metamorphosis. The resulting oozooid produces, by blastogenesis, one or two buds from the lateral wall that eventually will develop into functional blastozooids which, in turn, generate new budlets. Colonies contain many blastozooids arranged in star-shaped systems, embedded in a common tunic and interconnected by a plexus of blood vessels. Colonies undergo typical blastogenetic cycles in which all the zooids of the same generation are synchronised. Between 18 and 20 °C each generation of adult filter-feeding zooids lasts about one week (Sabbadin, 1969) and is subsequently resorbed during the takeover which is characterised by massive involution of the adult tissues (Burighel and Schiavinato, 1984). At the end of the process, primary buds open their oral and atrial siphons and replace the old zooids. Apoptosis is responsible for the death of the majority of the internal tissues of senescent zooids (Lauzon et al., 1992, 1993; Cima et al., 2003).

In the present report we investigated, using the fluorescent terminal dUTP nick end labelling (TUNEL) protocol coupled with classical histochemical technique (haematoxylin/eosin stain), the occurrence of apoptosis in *Botryllus schlosseri* throughout blastogenetic development, in the normal tissue turnover of adult zooids and during takeover. The presence of apoptotic cells was also investigated with electron microscopy (EM) for a further confirmation of the occurrence of the process evidenced by TUNEL. In consideration that it is known that the takeover stage is characterised by massive apoptosis (Lauzon et al., 1992, 1993, 2000, 2002; Cima et al., 2003), regressing zooids were used as positive control to evaluate the confidence of our experimental protocol.

Results indicate that apoptosis does not play a crucial role during blastogenesis, whereas it seems important in regulating adult tissue homeostasis and modulating the growth of the whole colony.

2. Material and methods

2.1. Animals

Colonies of *Botryllus schlosseri* (Stolidobranchia, Styelidae) were collected in the lagoon of Venice, let to adhere to glass slides, kept in aquaria at 18 °C and fed with Liquifry Marine (Liquifry Co., Dorking, England). Stages of colonial life cycle were defined according to Sabbadin (1955). Blastozooids development can be subdivided in various stages beginning with the appearance of the bud primordium (stage 1), continuing with the various steps of organogenesis, included the first heart beating (stage 8), opening of the

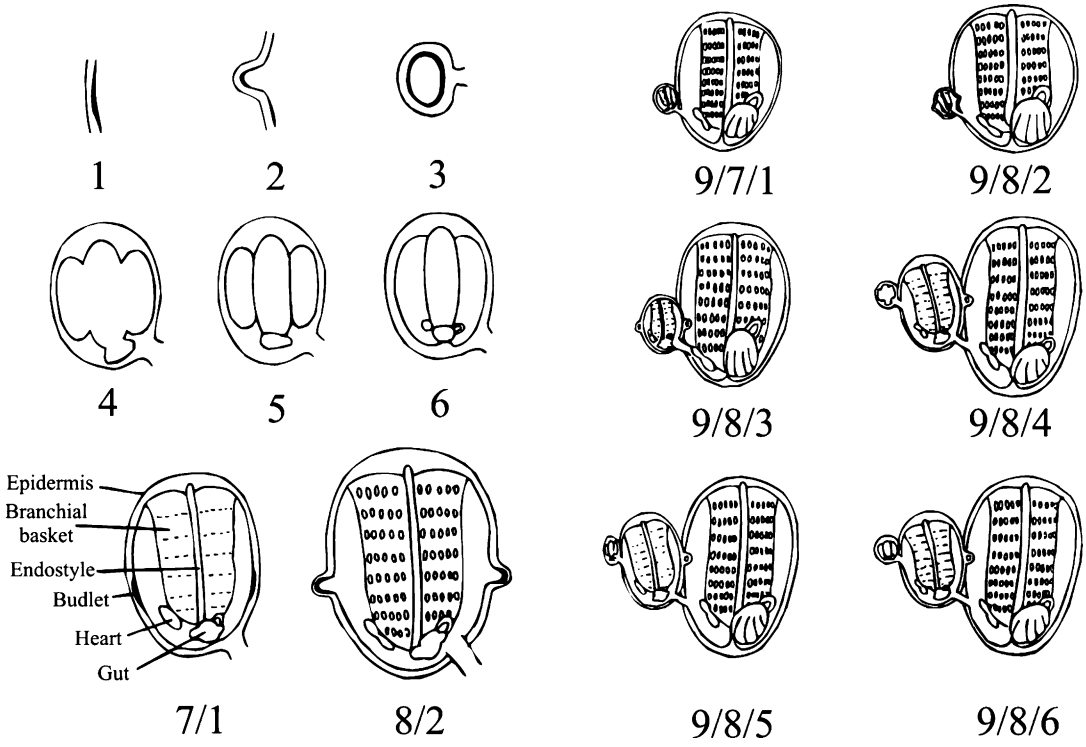


Fig. 1. Schematic drawing of the stages of blastogenetic development of *Botryllus schlosseri* according to Sabbadin (1958). Numbers indicate the developmental stage of budlets, buds and adults. A new budlet appears as a thickening of the bud mantle (stage 1) and develop up to stage 6. At stage 7, it becomes a bud able to give rise to new budlets; at stage 8 heart starts beating and at stage 9 the zooid opens its siphons and becomes functional. Since the development of the blastogenetic generations is highly synchronised, the stage of the colonial life cycle can be indicated by three numbers, indicating the developmental stage of each generation (e.g., 9/7/1). Drawings represent views of adults, buds and budlets, as seen from the ventral side. Only right buds of adults are shown.

siphons (stage 9), and ending with the takeover phase (stage 11). Since the development of buds and zooids is highly synchronised and three blastogenetic generation coexists in a colony, the stage of the colonial life cycle can be indicated by three numbers corresponding to the stage of each generation: 9/7/1, 9/8/2, 9/8/3, etc., the first number refers to the adult zooids, the second to buds and the third to budlets (Fig. 1). Investigated generation are marked by underlined numbers (i.e. 9/8/2). Stages 9/8/2 to 9/8/4 are considered mid-cycle stages as they are intermediate between siphon opening and the takeover.

2.2. Histochemistry and TUNEL on section

At least five colonies for each stage of the colonial life cycle were isolated and anaesthetised with MS222 (Sigma). Specimen were then fixed for at least 3 h in Karnowsky's solution (paraformaldehyde 4%, glutaraldehyde 0.1%, sodium cacodylate 0.4 M; pH 7.4), dehydrated in ethanol and embedded in Paraplast (Sherwood Medical). Sections (4–7 μm) from samples in various orientations were permeabilised with 1% Triton X-100 in phosphate buffered saline (PBS; NaCl 0.13 M, KCl 2.7 mM, Na_2PO_4 10 mM, KH_2PO_4 1.7 mM; pH 7.4) and incubated for 5 min in Evans Blue (0.5% in PBS) in order to quench the natural green fluorescence of the tissues. Sections were then treated with 85–90% of TUNEL label solution and 10–15% enzyme solution (in situ Cell Death Detection kit; Roche), according to the manufacturer's instructions, and incubated for 1 h at 37 °C in the dark. After vigorous washing in PBS, they were stained for 4 min with 1 μg/ml DAPI (Sigma) in PBS, mounted with Vectashield (Vector Laboratories) and finally observed under the fluorescence microscope, wavelength (excitation) 420/490 nm (Leica Dialux 22). In reference control, slides were stained for 4 min with 1 μg/ml DAPI (Sigma) in PBS; TUNEL enzyme solution was omitted in negative controls. In other experiments, sections were stained with haematoxylin and eosin to reveal the condensed nuclei in degenerating cells.

2.3. Electron microscopy

Colonies at various stages were fixed in 1.5% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.4, plus 1.6% NaCl. After washing and postfixation in 1% OsO_4 in 0.2 M sodium cacodylate, specimens were dehydrated and embedded in Epon resin. Sections (60 nm) were stained with uranyl acetate and lead citrate. Micrographs were taken with a Hitachi H-600 electron microscope operating at 80 kV.

3. Results

3.1. Blastogenesis in Botryllus schlosseri

Three blastogenetic generations generally coexist in the same colony: the filtering adults, their buds, and budlets. Our histological observations agree with that reported by Sabbadin (1955). Usually, each bud gives rise to four budlets, two on each side of the body: in this case, a difference in the course of the development of budlets on the opposite side of the parental bud can be observed, with a delay in those budlets arising from the left side of the bud compared to the right ones. In the same way anterior budlets develop faster than the posterior ones (Figs. 1 and 2A and B).

The budlet primordium appears as a thickened discs of the mantle (stage 1), that progressively folds forming a hemispheres (stage 2). Then, the young budlet forms a double vesicle that remains connected to and receives blood from the parental bud (stage 3). As development proceeds, the wall of the inner vesicle folds and a central branchial chamber is formed, flanked by two symmetrical peribranchial chambers (stage 4). The gut rudiment appears at the dorsal posterior end of the branchial chamber (stage 5) and afterwards the branchial and peribranchial chambers become well delimited, and the intestine and the stomach wall well distinguishable (stage 6). Then, primordia of the stigmata begin to form and new bud primordia appear on the mantle: the budlet is now a bud (stage 7). The heart starts beating at stage 8; the buds open their siphons and filtration starts at stage 9. Sexual maturity is reached at stage 10 and takeover occurs at stage 11 (Fig. 1).

3.2. Apoptosis in buds

A low amount of apoptotic cells was recorded in buds and budlets. At the stage 9/7/1, budlets were observable as a thickening of the mantle (delimited by the peribranchial chamber epithelium and the overlaying epidermis) and did not show any response to the TUNEL assay. At stage 2, when budlets begin their evagination from the parental bud, TUNEL-positive cells were detectable only in the region of the inner vesicle formation (Fig. 2C). Budlets did not show any TUNEL-positive cell at stages 3–6, except for rare haemocytes located between the inner vesicle and the epidermis (Fig. 2D and E).

In parental buds at stage 7, apoptosis was detectable in scattered single cells of the epidermis and the walls of the peribranchial chambers (Fig. 2G); the same structures stained with haematoxylin and eosin presented same pattern of pyknotic nuclei. Buds at stage 8, showed scattered apoptotic cells on the epidermis, in the mantle and in the peribranchial chambers epithelia (Fig. 2H and I). These kinds of responses were detectable throughout the stage 8 (from stages 9/8/2 to 9/8/4 of the colonial life cycle)

During normal blastogenetic cycle, each bud can generate up to four budlets (two posteriors and two anterior). Normally only two of them complete development, whereas the others degenerate and are usually reabsorbed at the stage 3 or 4 (Fig. 2F) (Sabbadin, 1955, 1966). Numerous apoptotic cells were detected in these budlets, and the degenerating cells were homogeneously distributed in the whole budlets (Fig. 2E).

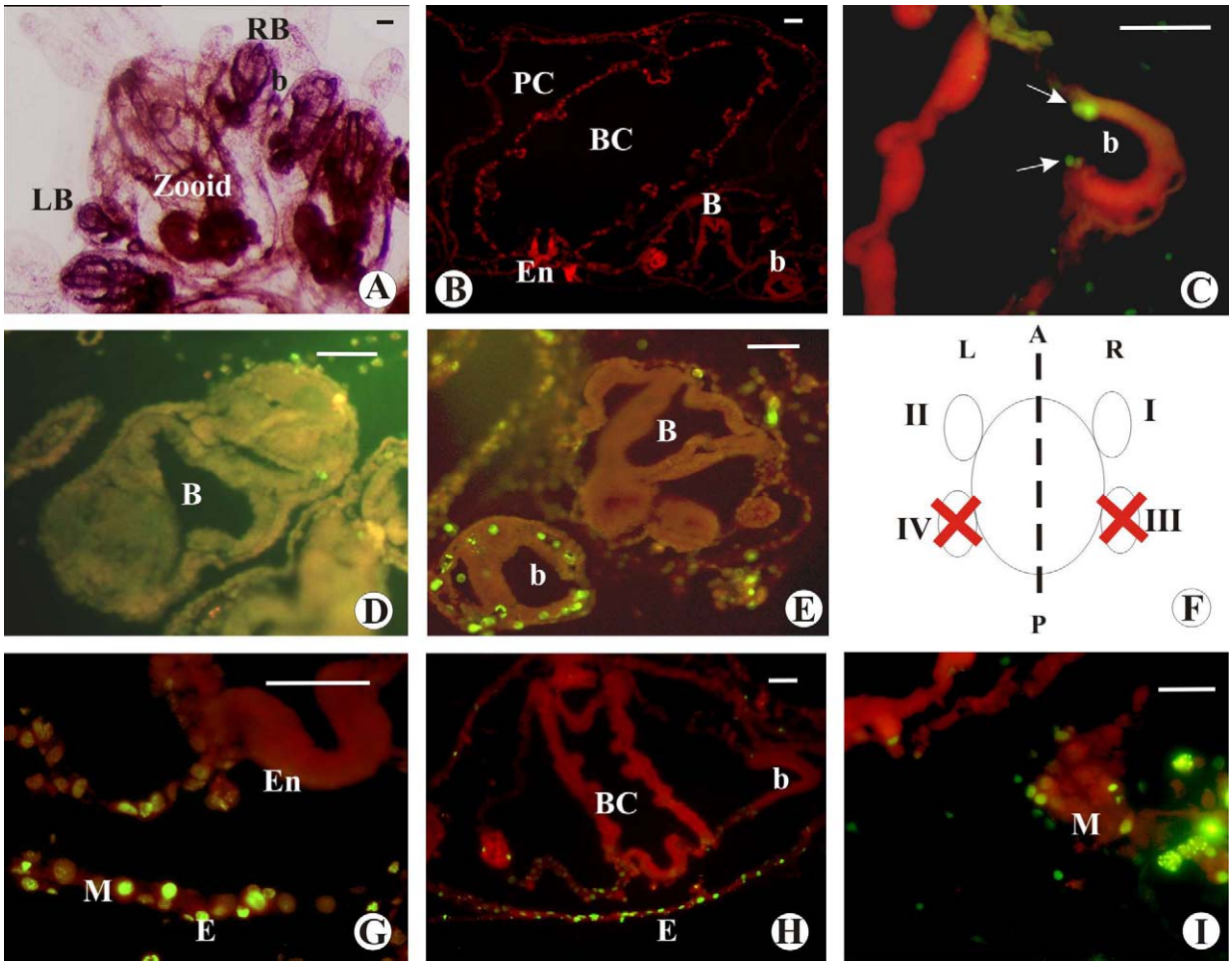


Fig. 2. (A) Particular of a whole-mounted colony of *Botryllus schlosseri* at stage 9/8/2 stained with haematoxylin and eosin; dorsal view. (B) Transversal section of a colony stained with Evans Blue (red); the image shows an adult zooid, its bud and the budlet on the bud. (C) Transversal section of a bud at stage 2 marked with TUNEL/Evans Blue; arrows indicate TUNEL-positive nuclei. (D) Bud at stage 5 showing no TUNEL-positive nuclei. (E) Bud at stages 6 and 7 and degenerating bud with scattered apoptotic nuclei. (F) Hierarchy of appearance of the buds (Roman numerals): in suboptimal environmental conditions only one (I) or two (II) buds completes its development; the others (III and IV) stops their growth and degenerate by apoptosis, as shown in figure (E). (G) Detail of peribranchial epithelium and epidermis (E) of a zooid at stage 7. (H) Transversal section of a blastozooid at stage 8 with a secondary bud at stage 2 set on the ventral part. (I) Detail of the mantel in stage 8. TUNEL-positive nuclei are marked in green whereas all negative tissues appear stained in red (Evans Blue); B: bud; b: budlet; BC: branchial chamber; E: epidermis; En: endostyle; LB: left bud; M: mantle; PC: peribranchial chamber; RB: right bud. Scale bars: 50 μm.

3.3. Apoptosis in adult tissues

Adult filtering zooids (stage 9) showed scattered labelled cells in epidermis, branchial and peribranchial leaflet and mantle (Fig. 3A and B). Presence of scattered TUNEL-positive cells was also found in the tunic as well as in circulating haemocytes and in cells of the vessel epithelium (Fig. 3C).

Massive apoptotic events were detected at the level of the male gonads of sexual mature zooids (stage 10): degenerative patterns were particularly abundant in the side of the gonad facing the stomach in the form of a crescent-shaped cluster of TUNEL-positive cells (Fig. 3D).

In oesophageal, gastric and intestinal epithelia of the filtering adult, the presence of sparse nuclei with condensed chromatin was observed (Fig. 4A–C). From random ultrastructural observations, the presence of scattered collapsing cells throughout the gut epithelium was detected in the adult, but also in the bud approaching the opening of the siphons (Fig. 4D and E). TUNEL-positive cells seem to be absent in the two regions constituted by bands of mucous cells (Fig. 4C). Phagocytes were not seen in filtering adults to infiltrate the gut epithelium. TUNEL positive cells were detected in the perivisceral epithelium, which border the stomach and the intestine and in blood lacunae between the perivisceral and the gut epithelia.

A particularly abundant presence of positive cells was observed in the ampullae of the pyloric gland (Fig. 4F and G). This structure is formed of a network of anastomising tubules ending in ampullae, which adhere to and encrust the epithelium of the middle intestine. Degenerative changes were diffuse in the gland epithelium especially at the level of the ampullae. These degenerative changes were not observed in buds and began to be commonly recognisable in blasto-

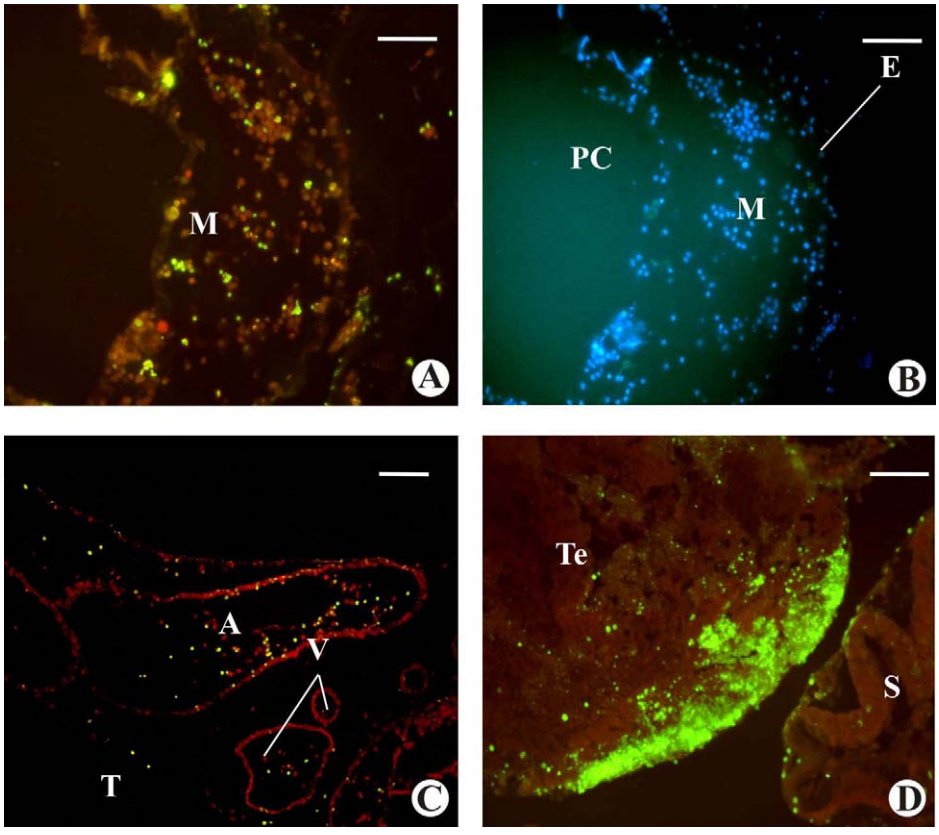


Fig. 3. (A and B) Detail of a section of the mantle in an adult blastozooid (stage 9) stained with TUNEL/Evans Blue and DAPI, respectively. (C) Vessels and ampulla embedded in the tunic (stage 9/8/4). (D) Detail of the male gonad of an adult zooid, all the tissues are stained by Evans Blue; fluorescent nuclei characterise TUNEL-positive cells. A: ampulla; E: epidermis; M: mantle; PC: peribranchial chamber; S: stomach; T: tunic; Te: testis. Scale bars: 50 μm.

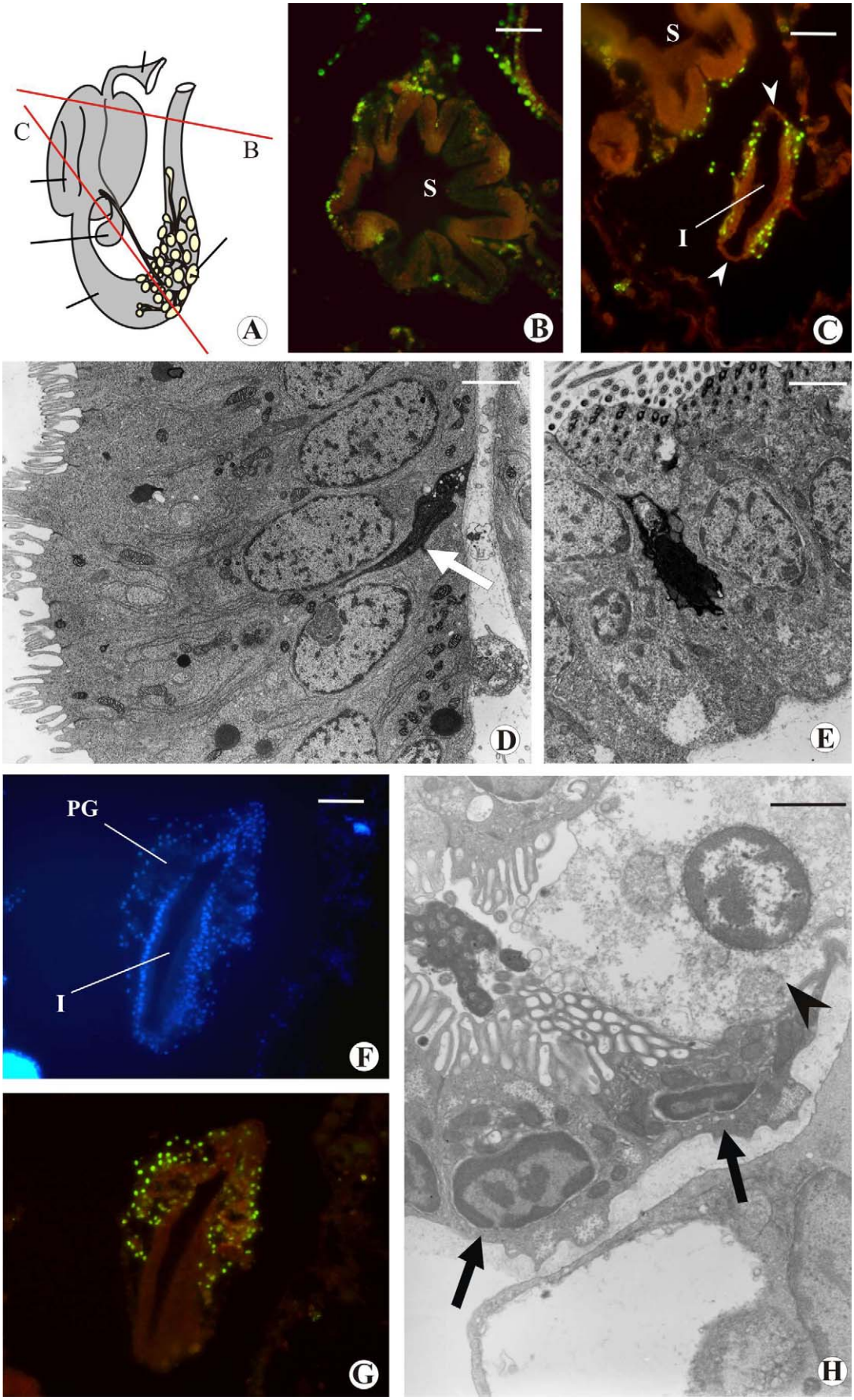
zooids approaching siphon opening. Electron microscopy confirmed the presence of cells with typical apoptotic features; moreover, others cells appeared swollen and lysed completely, leaking out their cytoplasm and organelles in the lumen (Fig. 4H). However, despite the fragmentation of cells, their thick basal lamina was maintained. The data recall the situation previously described in *B. schlosseri* (Burighel and Milanesi, 1977; Murre and Thouveny, 1977).

3.4. Apoptosis during takeover

A diffuse presence of TUNEL-positive nuclei characterise the takeover stage of the colonial life cycle; at the end of the takeover, TUNEL-positive nuclei appeared distributed in all the tissues of contracting and collapsing zooids (Fig. 5A and B). Particularly high rate of apoptotic nuclei was detected in the epithelium of the gut and in the pyloric gland. As previously described (Burighel and Schiavinato, 1984), cell fragments or the entire cells were engulfed by wandering phagocytes or by epithelial cells that act as fixed occasional phagocytes (Fig. 5C and D). Electron micrographs of the resorbing gut epithelia showed frequently aspects of cellular fragmentation or condensation of nuclei and cytoplasm, as the cells became roundish and retracted among the other epithelial cells (Fig. 5C).

4. Discussion

In Tunicata, the role of PCD by apoptosis has been studied during embryology, larval development and metamorphosis of solitary ascidians (Chambon et al., 2002; Jeffery, 2002a,b; Bates, 2004; Tarallo and Sordino, 2004) and during takeover of the colonial ascidian *Botryllus schlosseri*, when a gradual contraction occurs in regressing blastozooids and their tissues and organs undergo progressive apoptosis (Lauzon et al., 1992; Cima et al., 2003). Diffuse PCD by apoptosis characterises ascidian embryogenesis as morphogenetic cell death is required for the removal of the excess of cells. Conversely, scanty experimental data are available on the role of the same process during ascidian asexual development. In this contest, colonial ascidians represent ideal model organisms to compare the role of apoptosis during embryogenesis and blastogenesis as these two developmental pathways, although different (Tiozzo et al., 2005) converge to a common end product represented by the adult, filter-feeding zooid. In ascidians, during asexual reproduction zooids originate from an heterogeneous cell population instead of a single zygote so that we may assume that the equilibrium among proliferation, differentiation and cell death is regulated differently in blastogenesis and in embryogenesis. The present report considers PCD in the vegetative life cycle of the colonial ascidian


C
B
A
S
B
S
I
C
D
E
PG
I
F
G
H

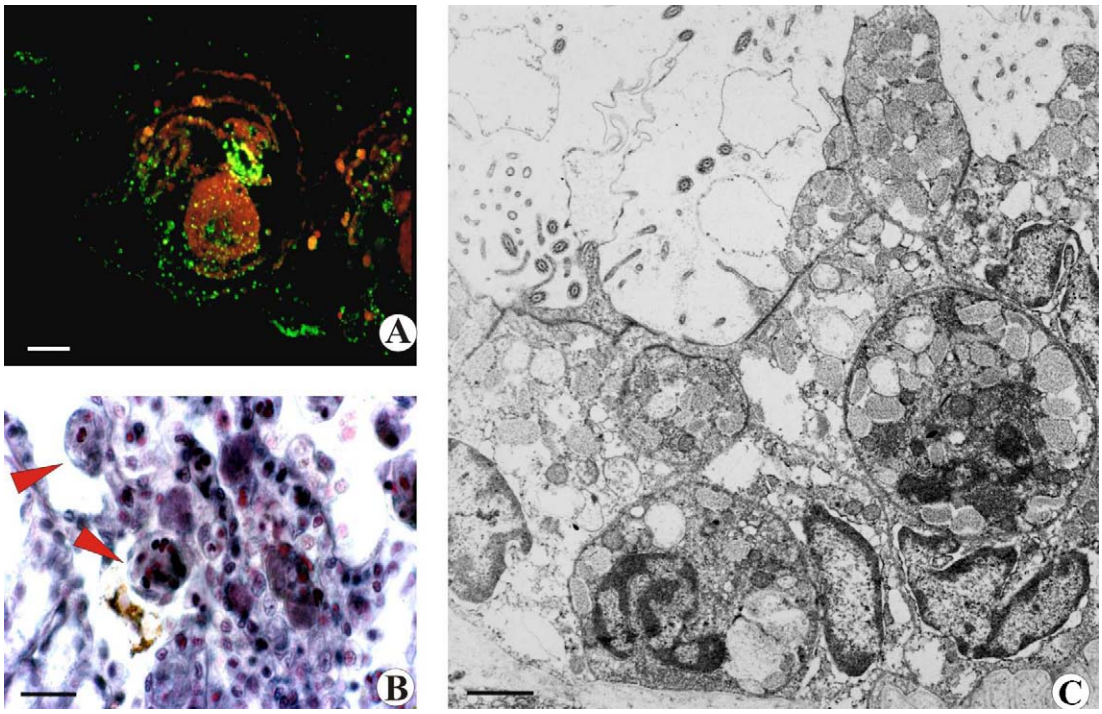


Fig. 5. (A) Collapsing adults during late takeover (stage 11) stained with TUNEL and Evans Blue, showing condensed apoptotic cells interspersed among epithelial cells. (B) Intestine stained with haematoxylin and eosin: apoptotic cells present characteristic pyknotic nuclei. Large circulating phagocytes (arrows) are engulfing senescent cells. (C) Details of oesophageal epithelium at late stage of regression. Scale bar: 50 μm for A; 15 μm for B; 1 μm for C.

Botryllus schlosseri throughout blastogenetic development from bud formation to the filter-feeding adult zooid.

Apoptotic cells were highlighted using fluorescent TUNEL staining, which is based on the presence of chromatin endonucleolysis, one of the key biochemical event of apoptosis. DNA polymerases as well as terminal deoxynucleotidyl transferase have been used for the incorporation of labelled nucleotides to DNA strand breaks that give a green fluorescence to the TUNEL-positive cells, whereas only red fluorescence (Evans Blue) is present in all of the tissues and in non-apoptotic nuclei. Further evidence of cell death was obtained by staining the same sections with haematoxylin/eosin and then looking for patterns of condensed chromatin that fit an event of apoptotic DNA fragmentation. An ultra-structural analysis of cell morphology was carried out using transmission electron microscopy on buds, filtering and regressing zooids. Apoptotic figures were found extensively during takeover and in some organs and tissues (i.e. gut and pyloric gland) of filtering adults, whereas they were scarce in buds.

In earlier developmental stages the role of apoptosis on organogenesis seemed rather marginal. Notable exception were reported during the double vesicle stage (stage 2). In this phase, when the inner vesicle, originating from evagination of the peribranchial wall, begins to detach from the parent, we found programmed death of cells, situated in the region connecting the young budlet to its parental epithelium. Lack of data about proliferation pattern during blastogenesis does not permit the formulation of a definitive hypothesis. Nevertheless our results suggest that, apoptosis could play a pivotal role in sculpturing the early budlet and assisting its detachment from the peribranchial leaflet of the parent.

During later blastogenetic stages, apoptosis was also found in some circulating cells in the primordium of the mantle. These haemocytes are not specific to buds or budlets, but come from the general circulation of the colony, where a constant rate of cell death is found even in stages far from the takeover.

The absence of apoptotic cells in buds at later blastogenetic stages (3–6) indicate that during most of the key

Fig. 4. (A) Alimentary post-branchial digestive tract of adult zooids. The red lines give the position of the sections (B) and (C), at the level of the stomach and the intestine, respectively; the two lateral bands marked by arrowheads consist of lower mucous cells. B and C were stained with TUNEL (green) and Evans blue (red). (D) Detail of gastric epithelium of an almost adult zooid (between stages 8 and 9) showing the presence of a dense degenerating cell (arrow). (E) A dense senescent cell in the oesophagus epithelium. (F and G) section at the level of the pyloric gland encrusting the intestine stained with DAPI (blue) and TUNEL (green)/Evans Blue (red), respectively: massive number of TUNEL-positive cells in the gland ampullae mirrors the high rate of degeneration of the gland. (H) Ultrastructural details of a pyloric gland showing the presence of cells with typical apoptotic features (arrows) close to a cell with clear signs of lysis in its cytoplasm (arrowhead). I: intestine; PG: pyloric gland; S: stomach. Scale bar: 50 μm for B, C, F and G; 1 μm for D, E and H.

morphogenetic events, such as the shaping of the branchial and peribranchial chambers, the perforation of the stigmata and the differentiation of the gut, no relevant PCD occurs. More conspicuous apoptotic episodes started to occur in stages 7 and 8, in which positive cells were scattered throughout the peribranchial and epidermis epithelia. Giving the rapid rate of growth that characterise these stages, we can suppose the presence of a high pattern of cell proliferation. In this perspective the low but constant presence of apoptosis could be considered as a mechanism of fine tuning of bud shaping during its growth. It is noteworthy that, differently from what reported for embryogenesis and larval development (Jeffery, 2002a,b; Bates, 2004), no apoptosis was observed during development of the nervous system. This agrees with the observation of Zaniolo et al. (2002) who reported that the final arrangement of neurons in the cerebral ganglion and nerves and their reduction in number occurs without participation of apoptosis.

Considering the adult zooid at stage 9, we reported scattered apoptotic events along the digestive tract and related structures. In these mid-cycle filtering blastozooids, such degenerative events could be considered part of a normal tissue turnover. The intermittent nature of the cell death makes an ultrastructural analysis difficult, however isolated collapsing cells have been found in both stomach and intestine epithelia. The presence of wandering phagocytes involved in engulfment of apoptotic cells was not observed in filtering zooids despite a great number of circulating phagocytes in the blood. In contrast, during zooid takeover, phagocytosis of apoptotic bodies and senescent cells by circulating phagocytes and also by neighbouring epithelial cells was frequented observed (Burighel and Schiavinato, 1984).

Of particular interest is the pattern of degeneration occurring at the level of the pyloric gland, which adheres to intestine, but opens its duct into the stomach. Participation of this structure in sugar metabolism and glycogen storage has been documented, so that its glandular cells are reminiscent of the hepatocytes of the vertebrate liver (Mugnaini and Harboe, 1967; Ermak, 1977; Burighel and Cloney, 1997). Moreover, digestion, osmoregulation and excretory functions have been reported (Burighel and Cloney, 1997), even if the role of the gland in the zooid physiology is still uncertain. Our observations show constant and diffuse TUNEL-positivity in this structure throughout the entire blastogenetic cycle. The degeneration of the blind gland ampullae, adhering to the intestinal epithelium, seems to occur not only by apoptosis but also by cytolysis, as revealed by both typical apoptotic features and the presence of necrotic cells and debris which are not engulfed by phagocytes, but are discarded into the lumen and eliminated throughout the duct.

Gonads appear early in the young blastozooids; nevertheless environmental conditions strongly influence the sexual reproduction and, under stress situations, mature blastozooids can reabsorb their gonads (Sabbadin, 1955). Thus, gonad involution is not uncommon and testis and ovary can disappear for several blastogenetic generations. Through this strategy, a stressed colony saves energy and resources. Our results clearly show that, at least for testis, gonads degenerate by diffuse apoptosis. A similar process of PCD was described in the ovary of *Boltenia villosa* (Bates, 2004). Driven by the same principle of economy only one or two budlets, out of four, originate by the parental bud, complete their development in suboptimal conditions (Sabbadin, 1955, 1956). In response to stress situations, secondary buds, usually grown up to stages 3 and 4, stop their development and are gradually reabsorbed. Here we demonstrate that apoptosis is the mechanism that zooids adopt to regulate the number of their buds and, as a consequence, the homeostasis of the whole colony. In this context the use of PCD could be seen in a strictly ecological perspective as a tool to adapt colony growth to the environment. In contrast, PCD during the takeover represents a predictable, periodic and synchronised event for the renewal and substitution of colonial components, the regulation of which is probably under genetic control. Our data on the senescent phase of the colonial life cycle confirm the observations of Lauzon et al. (1992, 1993) and Cima et al. (2003). From another point of view, this stage provides a good control for comparison of the other phases of the colonial life cycle.

In conclusion, in contrast to sexual development (embryogenesis and metamorphosis) (Jeffery, 2002a,b; Chambon et al., 2002; Bates, 2004; Tarallo and Sordino, 2004) during vegetative propagation in botryllid ascidians apoptosis does not seem to play an important role. However, PCD emerges as a key force to regulate tissue homeostasis in adult zooids and to shape and modulate the growth of the whole colony. Further observation of the role of apoptosis in the compound ascidian *Botryllus schlosseri*, coupled with the analysis of proliferative profile may provide a means to understand how growth, differentiation and death are coordinated and organised in colonial organisms and how these mechanisms are regulated by environmental factors.

Acknowledgements

This research was supported by the Italian MIUR (PRIN 2003 and 2004). Thanks are due to Mr. M. Del Favero and Mrs. C. Boeche for technical help. We also thank Prof. Roger Croll for discussion and critical review of the manuscript.

References

Aravind, L., Dixit, V.M., Koonin, E.V., 1999. The domains of death: evolution of the apoptosis machinery. Trends Biochem. Sci. 24, 47–53.

Bates, W.R., 2004. Cellular features of an apoptotic form of programmed cells death during the development of the ascidian, *Boltenia villosa*. Zool. Sci. 21, 553–563.

Burighel, P., Milanesi, C., 1977. Fine structure of the intestinal epithelium of the colonial ascidian *Botryllus schlosseri*. Cell Tissue Res. 182, 357–369.

Burighel, P., Schiavinato, A., 1984. Degenerative regression of the digestive tract in the colonial ascidian (*Botryllus schlosseri* Pallas). Cell Tissue Res. 235, 309–318.

Burighel, P., Cloney, R.A., 1997. Urochordata: ascidiacea. In: Harrison, F.W., Ruppert, E.E. (Eds.), Microscopic Anatomy of Invertebrates, vol. 15. Wiley–Liss, New York, pp. 221–347.

Chambon, J.P., Soule, J., Pomies, P., Fort, P., Sahuquet, A., Alexandre, D., Mangeat, P.H., Baghdiguian, S., 2002. Tail regression in *Ciona intestinalis* (Prochordate) involves a caspase-dependent apoptosis event associated with ERK activation. Development 129, 3105–3114.

Cikala, M., Wilm, B., Hobmayer, E., Bottger, A., David, C.N., 1999. Identification of caspases and apoptosis in the simple metazoan *Hydra*. Curr. Biol., 959–962.

Cima, F., Basso, G., Ballarin, L., 2003. Apoptosis and phosphatidylserine-mediated recognition during the take-over phase of the colonial life-cycle in the ascidian *Botryllus schlosseri*. Cell Tissue Res. 312, 369–376.

Cole, A.G., Meinertzhagen, I.A., 2004. The central nervous system of the ascidian larva: mitotic history of cells forming the neural tube in late embryonic *Ciona intestinalis*. Dev. Biol. 271, 239–262.

Ermak, T.H., 1977. Glycogen deposits in the pyloric gland of the ascidian "*Styela clava*" (Urochordata). Cell Tissue Res. 176, 47–55.

Gordon, D.P., 1977. The aging process in bryozoans. In: Woollacott, R.M., Zimmer, R.L. (Eds.), Biology of Bryozoans. Academic Press, New York, pp. 335–376.

Jeffery, W.R., 2002a. Programmed cell death in the ascidian embryo: modulation by FoxA5 and Manx and roles in the evolution of larval development. Mech. Dev. 118, 111–124.

Jeffery, W.R., 2002b. Role of PCNA and ependymal cells in ascidian neural development. Gene 287, 97–105.

Koonin, E.V., Aravind, L., 2002. Origin and evolution of eukaryotic apoptosis: the bacterial connection. Cell Death Differ. 4, 394–404.

Kott, P., 1989. Form and function in the Ascidiacea. Bull. Mar. Sci. 45, 253–276.

Lauzon, R.J., Ishizuka, K.J., Weissman, I.L., 1992. A cyclical, developmentally-regulated death phenomenon in a colonial urochordate. Dev. Dynam. 194, 71–83.

Lauzon, R.J., Patton, C.W., Weissman, I.L., 1993. A morphological and immunohistochemical study of programmed cell death in *Botryllus schlosseri* (Tunicata, Ascidiacea). Cell Tissue Res. 272, 115–127.

Lauzon, R.J., Rinkevich, B., Patton, C.W., Weissman, I.L., 2000. A morphological study of nonrandom senescence in a colonial urochordate. Biol. Bull. 198, 367–378.

Lauzon, R.J., Ishizuka, K.J., Weissman, I.L., 2002. Cyclical generation and degeneration of organs in a colonial urochordate involves crosstalk between old and new: a model for development and regeneration. Dev. Biol. 249, 333–348.

Mugnaini, E., Harboe, S.B., 1967. The liver of *Mixine glutinosa*: a true tubular gland. Z. Zellfor. 78, 341–369.

Murre, C., Thouveny, Y., 1977. Etude ultrastructurale de la glande pylorique de l'ascidie "*Botryllus schlosseri*". Publ. Bull. Soc. Zool. France 102, 439–443.

Namba, R., Pazdera, T.M., Cerrone, R.L., Minden, J.S., 1997. *Drosophila* embryonic pattern repair: how embryos respond to bicoid dosage alteration. Development 124, 1393–1403.

Sabbadin, A., 1955. Osservazioni sullo sviluppo, l'accrescimento e la riproduzione di *Botryllus schlosseri* (Pallas) in condizioni di laboratorio. Boll. Zool. 22, 243–263.

Sabbadin, A., 1956. Osservazioni sull'accrescimento delle gemme e degli zooidi di *Botryllus schlosseri* (Pallas) [Ascidiacea], in condizioni normali e sperimentali. Rend. Accad. Naz. Lincei 20, 485–491.

Sabbadin, A., 1958. Analisi sperimentale dello sviluppo delle colonie di *Botryllus schlosseri* (Pallas). Arch. It. Anat. Zool. 18, 178–221.

Sabbadin, A., 1966. Aspetti e problemi della vita coloniale. Boll. Zool. 33, 23–38.

Sabbadin, A., 1969. The compound ascidian *Botryllus schlosseri* in the field and in the laboratory. Pubbl. Staz. Zool. Napoli 37, 62–72.

Tarallo, R., Sordino, P., 2004. Time course of programmed cell death in *Ciona intestinalis* in relation to mitotic activity and MAPK signaling. Dev. Dynam. 230, 251–262.

Tiozzo, S., Christiaen, L., Deyts, C., Manni, L., Joly, J.-S., Burighel, P., 2005. Embryonic versus blastogenetic development in the compound ascidian *Botryllus schlosseri*: insights from *Pitx* expression patterns. Dev. Dynam. 232, 468–478.

Zahir, N., Weaver, V.M., 2004. Death in the third dimension: apoptosis regulation and tissue architecture. Curr. Opin. Genet. Dev. 14, 71–80.

Zaniolo, G., Lane, N.J., Burighel, P., Manni, L., 2002. Development of the motor nervous system in ascidians. J. Comp. Neurol. 443, 124–135.