Chapter 2

Studying Regeneration in Ascidians: An Historical Overview

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Abstract

Ascidians are sessile tunicates, that is, marine animals belonging to the phylum Chordata and considered the sister group of vertebrates. They are widespread in all the seas, constituting abundant communities in various ecosystems. Among chordates, only tunicates are able to reproduce asexually, forming colonies. The high regenerative potentialities enabling tunicates to regenerate damaged body parts, or the whole body, represent a peculiarity of this taxon. Here we review the methodological approaches used in more than a century of biological studies to induce regeneration in both solitary and colonial species. For solitary species, we refer to the regeneration of single organs or body parts (e.g., siphon, brain, gonad, tunic, viscera). For colonial species, we review a plethora of experiments regarding the surgical manipulation of colonies, the regeneration of isolated colonial entities, such as single buds in the tunic, or part of tunic and its circulatory system.

Key words Colonial circulatory system, Evisceration, Gonad, Neural complex, Partial regeneration, Siphon, Thorax, Tunic, Whole body regeneration

1 Introduction

Within the phylum Chordata, which includes the three subphyla Vertebrata, Cephalochordata, and Tunicata (Fig. 1), the latter exhibits the more striking regenerative abilities. This feature, widely recognized by the scientific community since the end of the nineteenth century, raised renewed interests in the last 15 years, thanks to the availability of new methodological tools enabling the dissection of its molecular and cellular bases [1, 2]. In tunicates, the regenerative ability shows remarkable differences in various clades, even in different tissues and organs of the same organism [3]. Nonetheless, in some species, it can extend to extremes of complete individuals formed, from a small group of stem cells in the case of whole-body regeneration (WBR) [4, 5].

Tunicates are filter feeding, marine organisms, widespread in all the seas, constituting abundant communities in various ecosystems.

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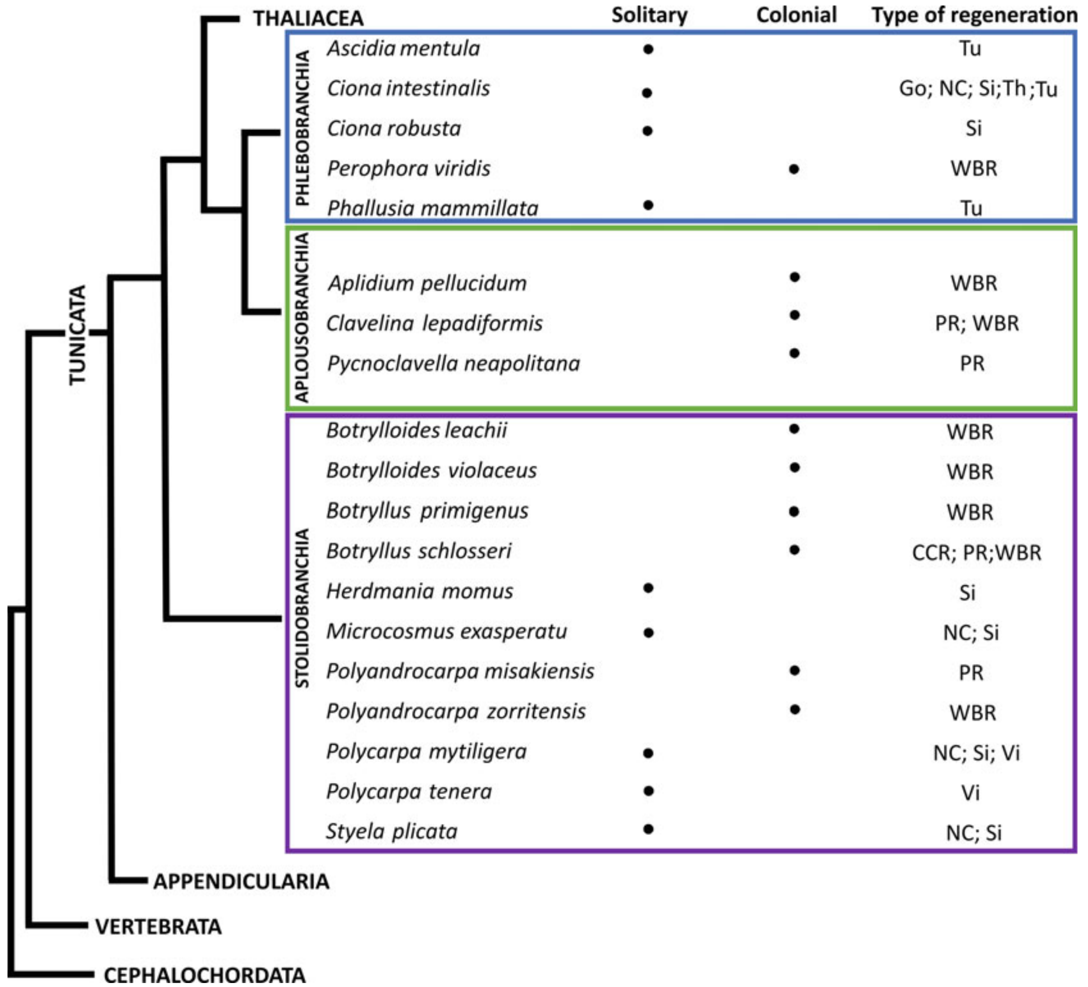

Fig. 1 Phylogenetic tree of chordates (modified from [7]) reporting the ascidian species studied for regeneration. Dots indicate if species are solitary or colonial. Types of regeneration induced in the various species include the following: regeneration of the colonial circulatory system (CCS), gonads (Go), neural complex (NC), partial regeneration (PR), regeneration of the siphons (Si), thorax (th), tunic (tu), viscera (Vi). WBR: whole body regeneration

They are considered the sister group of vertebrates (Fig. 1) [6, 7], therefore representing, from an evolutionary point of view, an intriguing taxon for comparative studies on regeneration, a limited process in vertebrates [8, 9]. Tunicates include both solitary and colonial species, the latter representing the only chordates capable of asexual reproduction (also called budding) [10].

Ascidians represent the main tunicate group, now considered paraphyletic by several authors. Their life cycle includes a swimming larva, which exhibits the typical chordate body plan and is the dispersal phase of the species. The larva is lecithotrophic and swims for a few hours to select an appropriate substrate on which

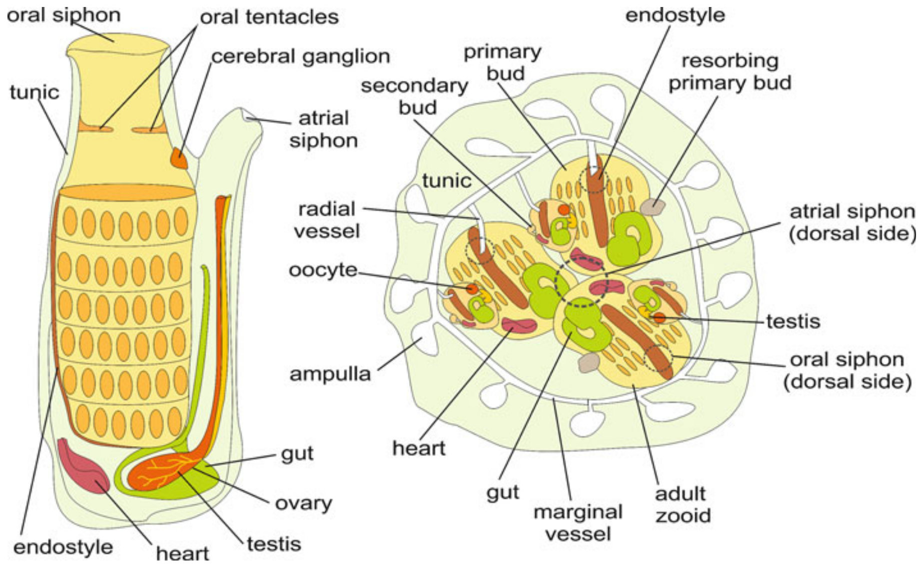

Fig. 2 Schematic representation of a solitary (left) and a colonial (right) ascidian (in ventral view). In the colonial tunicate, three adult zooids are represented, each one bearing one primary bud and one secondary bud

to adhere. Then, it undergoes a deep metamorphosis becoming a sessile individual, the juvenile, which possesses the capacity to regenerate.

Ascidian adult body is cylindrical, with an anterior inhalant (oral) siphon and a dorsal exhalant (atrial) siphon (Figs. 2 and 3). The brain (called cerebral ganglion) is located between the two siphons. The brain, together with the associated neural gland, forms the neural complex. The majority of the body is occupied by a large branchial chamber (pharynx) perforated by numerous ciliated stigmata [11]. The inhaled water enters the branchial chamber and, passing through the stigmata, is filtered by a mucous net produced by the endostyle. The latter is a glandular groove located in the ventral floor of the branchial chamber. The filtered seawater passes then to the atrial chamber and is expelled through the atrial siphon. Nutrients, entrapped in the mucous net, are agglutinated in a mucous cord that is conveyed to the U-shaped gut, located posteriorly, below the branchial chamber. The anus opens into the atrial chamber, so that the fecal pellets are removed by the exhalant water flow. Ascidians are hermaphrodites; gonads can be located in the posterior body, close to the gut, or in the lateral body wall.

In solitary ascidians, the cylindrical, tube-like body shape suggested a wide range of regeneration experiments involving mainly distal body parts, such as the siphons and the neural complex (Table 1) (Fig. 3b–f). In the solitary species of the genus *Ciona*, *C. intestinalis* and *C. robusta*, for which very advanced methodological tools are available, regeneration has been comprehensively

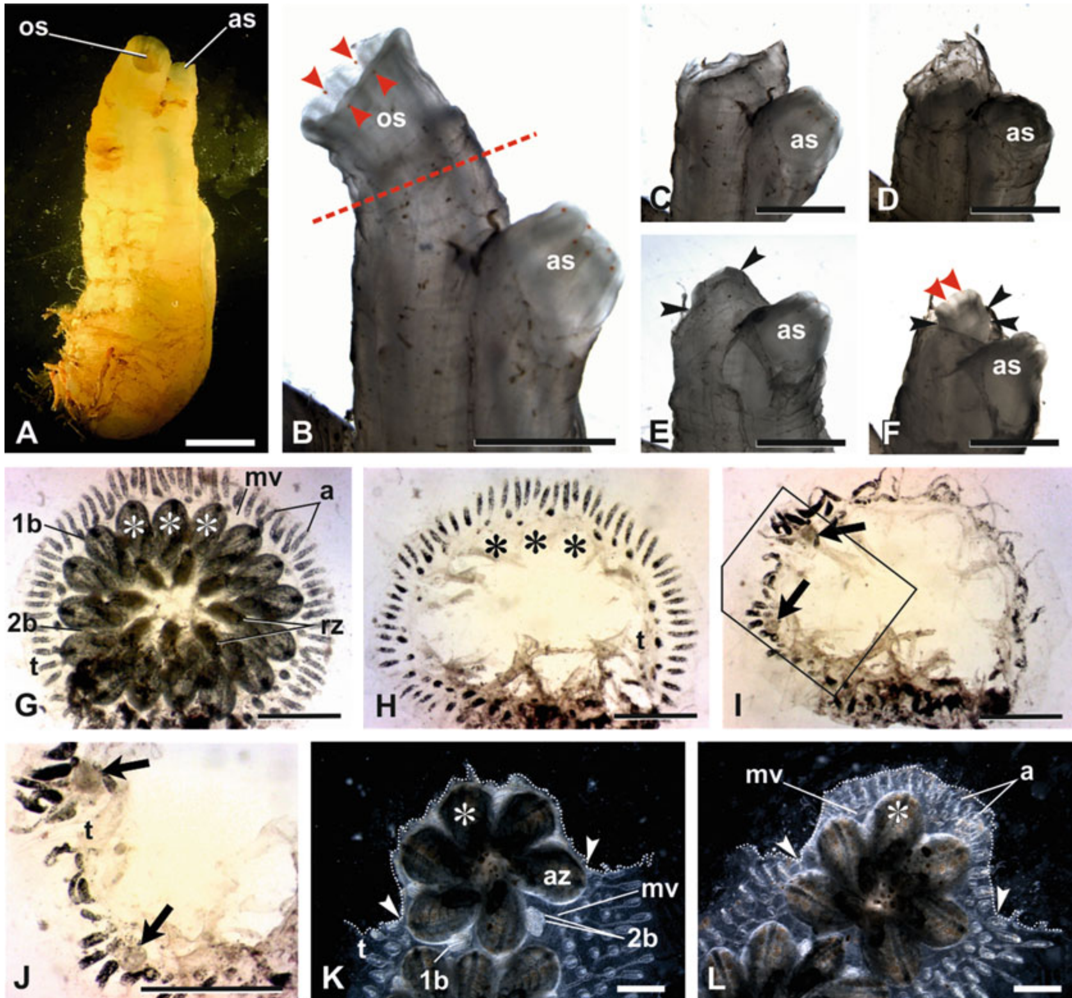

Fig. 3 (**A**–**F**). Oral siphon regeneration in *Ciona robusta*. (**A**) Individual showing the typical cylindrical, tube-like body shape of solitary ascidians. The oral siphon (os) individuates the anterior side, the atrial siphon (as) the dorsal side. (**B**–**F**). Details of siphons of an individual before (**B**), and after 0 (**C**), 1 (**D**), 3 (**E**), and 6 days (**F**) from the amputation of the oral siphon. The red line in **B** labels the level of amputation. One day after amputation (**D**), the wound is closed. In (**E**, **F**), black arrowheads mark the basal limit of the regenerating oral siphon. In (**B**) and (**F**), red arrowheads label some of the eight orange-pigmented sensory organs located in the notches between the lobes of the oral siphon rim. Six similar organs are also on the atrial siphon. Note that after 6 days from oral siphon ablation (**F**), the organs are present. (**G**–**J**). WBR in *Botryllus schlosseri*. In a colony at takeover phase (**G**; ventral view), regressing adult zooids (rz) are in form of dense masses at the center of the colony; primary buds (1b) are almost ready to open their siphons becoming the new generation of filtering individuals; small secondary buds (2b) are recognizable on primary buds. The colony is embedded by the tunic (t), where the colonial circulatory system is located. A marginal vessel (mv) extends all around the colony, connecting and coordinating the zooids. Blind ampullae (a) elongate from the marginal vessel toward the periphery. (**H**) colony (dorsal view) showed in **G** 2 days after the ablation of all the zooids (regressing zooids, primary and secondary buds). The circulation is restored. Asterisks in **H** individuate the position of the three primary buds marked by asterisks in **G**. (**I**–**J**) 3 days post ablation, two new vascular buds (arrows) are recognizable close to the marginal vessel. Square area in **I** is enlarged in **J**. (**K**–**L**) Tunic and colonial circulatory system regeneration in *Botryllus schlosseri* (ventral view). (**K**) a portion of the tunic (t) with its marginal vessel (mv) and blood ampullae has been removed (dotted line) in front of three adult zooids (az).

Table 1
Types of regeneration studies in solitary ascidians

Regenerating organ	Species	Outcome	References
Tunic	*Ascidia mentula*	Full regeneration	[19]
	Phallusia mammillata	Full regeneration	[20]
	Ciona intestinalis	Full regeneration	[23]
Thorax	*Ciona intestinalis*	Full regeneration only if fragments of the pharynx remains and in posterior toward anterior direction	[18]
	Polycarpa mytiligera	Full regeneration from posterior toward anterior direction	[14]
Siphon (oral/atrial)	*Ciona intestinalis*	Full regeneration (short/long distance regeneration for oral siphon)	[32, 33]
	Polycarpa mytiligera	Full regeneration	[13, 31]
	Styela plicata	Full regeneration	[31]
	Microcosmus exasperatus	Full regeneration	[31]
	Herdmania momus	Scrubby regeneration	[31]
Neural complex	*Styela plicata*	Full regeneration	[31, 70]
	Ciona intestinalis	Full regeneration	[16, 67–69]
	Microcosmus exasperatus	Full regeneration	[31]
	Polycarpa mytiligera	Full regeneration	[31]
Gonad	*Ciona intestinalis*	Full regeneration	[66]
Viscera	*Polycarpa mytiligera*	Full regeneration	[13, 31]
	Polycarpa tenera	Full regeneration	[12]
Body parts with multiple organs	*Polycarpa mytiligera*	Full regeneration of complete individuals from body fragments obtained by cutting animals longitudinally and transversely	[14]

Fig. 3 (continued) Arrowheads individuate the lateral cut edges. (**L**) the same colony showed in (**K**), 6 days after ablation: new tunic covers the previously exposed zooids. (**L**) in the regenerated tunic, the marginal vessel and a crown of new ampullae (a) are recognizable. Asterisks in **K** and **L** individuate the same zooid, as reference. Scale bar = 1 mm in **a**, (**G–J**); 5 mm in (**B–F**); 100 μ in (**K**, **L**).

explored in terms of both its morphological and cellular/molecular aspects (Table 2) [1]. Among other emerging model species, *Polyandrocarpa mytiligera* is a solitary ascidian of the Red Sea that, for example, can regenerate the whole gut after its evisceration [12–14].

In colonial ascidians, several individuals (zooids) are organized in large colonies exhibiting astonishing morphologies and colors (Fig. 3g) [10]. Usually, regeneration is seen as the ability of an organism to regrow or repair its cells, tissues and organs after their loss or severe injury. However, to some extent, asexual reproduction in colonial ascidians is considered an expansion of regeneration, as a nonembryonic development of new individuals. Indeed, the ability of colonial ascidians to activate unusual developmental pathways in both natural and/or induced conditions makes the border between asexual reproduction and a true, injury-induced, regeneration quite faint [2, 11].

In colonial ascidians, a plethora of regeneration experiments has been performed regarding, in general, the removal of single individuals from colonies (single buds, adult individuals, both buds and adult zooids) (Table 3; Fig. 3g–j) [2]. However, the regeneration of isolated colonial entities (e.g., isolated buds in the tunic, the whole tunic with its circulatory system without any zooid) and the regeneration of part of the tunic and its circulatory system have also been studied (Fig. 3k–l). The latter is a network of hemolymphatic vessels (Fig. 3g) connected to a marginal vessel that extends along the periphery of the colony. Several radial vessels emerge from each zooid and connect them to the marginal vessel. Blind, sausage-like ampullae elongate toward the tunic periphery. Among colonial ascidians, *Botryllus schlosseri* is one of the most studied species. However, several ascidians species can be maintained in laboratory culture throughout their life cycle and used for regeneration experiments (Table 3). Thanks to their recurrent budding, the ability to survive in aquaria also beyond their natural lifespan (useful to study ageing), the possibility to be split in fragments to analyze different conditions in the same genetic environment, their sequenced genome, and the availability of some molecular tools for unbiased results (Table 4), colonial ascidians provide valuable models for an integrated approach to regeneration.

On the whole, studies on both solitary and colonial species are shedding light in outstanding challenging topics of contemporary biological science, such as the connections between animal regeneration and regenerative medicine, stem cells biology, aging, and tissue homeostasis [1, 15].

Here, we briefly review the different types of regeneration experiments performed in ascidians in more than a century. Moreover, we present, in an historical perspective, the methodological approaches used to induce regeneration in both solitary and colonial ascidians.

Table 2
Experimental procedures used in the study of regeneration of solitary ascidians

Experimental procedure	Species	Methods	References
Tunic removal	*Ascidia mentula*	In vivo observations	[19]
	Phallusia mammillata	In vivo observations	[20]
	Ciona intestinalis	In vivo observations	[23]
Thorax	*Ciona intestinalis*	In vivo observations	[18]
Siphon (oral/atrial) ablation	*Ciona intestinalis*	In vivo observations	[80]
		Histological analysis	[32, 33]
		EdU labeling for proliferating cells	[32, 80]
		pH 3 labeling for proliferating cells	[80]
		Inhibition of cell proliferation with colchicine or nocodazole	[32]
		UV irradiation to study the involvement and the origin of stem cells	[80, 81]
		Anti-piwi antibody to label stem cells	[32]
		Alkaline phosphatase histoenzymology to reveal stem cells	[32]
		In vitro cultures of siphon explants	[80, 81]
		Distal, middle, basal, or oblique amputations to study the origin of the orange-pigmented sensory organ progenitor cells	[80]
		Branchial sac transplantation to assess the role of the pharynx as source of stem cells	[32, 80]
		Repeated ablation to study the effects of a possible depletion of stem cells	[80]
		Amputation in young and aged animals to study the effect of aging	[32, 81, 82]
		Treatment of animals in notch inhibitors to study the role of the notch pathway in regeneration	[83]
		Exposure of TGFβ inhibitors to study the involvement of TGFβ-mediated signal transduction in regeneration	[84]
		Use of transgenic animals to study the role of the nervous system in the process	[69]
		RNAseq of regenerating fragments to reveal differentially expressed genes	[83]
	Polycarpa mytiligera	In vivo observations	[13, 31]
		Histological analysis	[31]
	Styela plicata	In vivo observations and histological analysis	[31]
	Microcosmus exasperatus	In vivo observations and histological analysis	[31]
	Herdmania momus	In vivo observations and histological analysis	[31]

(continued)

Table 2 (continued)

Experimental procedure	Species	Methods	References
Neural complex ablation and brain chemical degeneration	*Styela plicata*	Induction of brain degeneration using 3-acetylpyridine and recovery study by means of behavioral experiments, TEM analysis, immunohistochemistry, Western blotting	[70]
	Ciona intestinalis	Histology, monoclonal antibodies, BrdU labeling, colchicine treatment	[67, 68]
		Live imaging and EdU labeling	[33, 80, 82]
		Use of old animals to study the effects of aging	[82]
		Live imaging, Nomarski images, confocal imaging, EdU incubations and staining for GFP, behavioral responses	[69]
Gonad removal	*Ciona intestinalis*	In vivo observation and histological analysis	[66]
Evisceration	*Polycarpa tenera*	In vivo observation and histological analysis	[12]
	Polycarpa mytiligera	In vivo observation and histological analysis	[13]
Dissection of individuals in fragments	*Polycarpa mytiligera*	In vivo observation, histological analysis, EdU labeling for proliferating cells, confocal imaging	[14]

2 Regeneration in Solitary Ascidians

The study of regeneration in solitary ascidians has a long story as it began at the end of the nineteenth and the early beginning of the twentieth century, with the experiments carried out by a series of German scientists working at the Stazione Zoologica, in Naples (Italy), newly founded by Anthon Dohrn [16–18]. With the establishment of a series of new French marine stations at the beginning of the last century, the contributions of the Belgian-French scientists appeared and, rapidly, acquired a predominance that was maintained throughout the first half of the century [19–24]. Then, the American school arose and, quickly, reached the visibility that it still has [25–27].

Solitary ascidians are capable of partial body regeneration (Table 1). In all the studied species, the epidermis can easily regenerate the tunic once the latter is removed [19, 20, 23]. When part of the body is removed, in most cases it can be reformed quite rapidly. *C. intestinalis* has been the reference model for the study of solitary ascidian regeneration for more than a century. In this species, when an animal is bisected, only the basal part can

Table 3
Types of regeneration studies in colonial ascidians

Type of regeneration	Species	Outcome	References
WBR			
Regeneration from colonial vasculature	*Botryllus schlosseri*	Vascular bud development	[5, 48–51]
	Botryllus primigenus	Vascular bud development	[45–47]
	Botrylloides leachii	Vascular bud development	[71, 73, 74, 76, 77]
	Botrylloides violaceus	Vascular bud development	[72]
	Botrylloides diegensis	Vascular bud development	[52]
Regeneration from zooid fragments	*Botryllus schlosseri*	Development of budlets from the remaining tissues	[50, 53, 54]
	Clavelina lepadiformis	Remodeling of remaining tissues and development of new buds	[21, 55]
	Aplidium pellucidum	Remodeling of remaining tissues and development of new buds	[39]
	Perophora viridis	Resorption of mature zooids and development of new buds	[39, 40]
Regeneration from postaddomen and stolon	*Clavelina lepadiformis*	New buds formation	[21]
Regeneration from isolated stolon fragments	*Polyandrocarpa zorritensis*	New buds formation	[56]
	Clavelina lepadiformis	New buds formation	[21, 36–38, 85–89]
	Perophora viridis	New buds formation	[25, 39, 40]
Partial regeneration			
Damaged bud regeneration	*Botryllus schlosseri*	Regeneration of the damaged parts	[27]
	Clavelina lepadiformis	Regeneration of damaged part from esophageal fragments	[21]
	Pycnoclavella neapolitana	Regeneration of damaged parts from the anterior end of the epicardium	[59]
	Polyandrocarpa misakiensis	Regeneration of amputated parts from the atrial epithelium	[60, 61]
Regeneration of the colonial circulatory system			
Colonial circulatory system regeneration	*Botryllus schlosseri*	Regeneration of vessels and ampullae	[62–65, 78]

regenerate the missing one, provided that part of the pharyngeal basket is conserved; conversely, the distal part is unable to regenerate any basal structure. The regeneration includes organs such as the siphons, the neural complex, the gonads and the missing part of the digestive system.

Table 4
Experimental procedures used in the study of regeneration of colonial ascidians

Experimental procedure	Species	Methods	References
WBR			
Isolation of colonial vasculature	*Botryllus schlosseri*	In vivo observations	[5, 48–50]
		Live imaging, microinjection of cells from isogenic colonies, circulation parameters measurements, histology	[5]
		Immunohistochemistry, in situ hybridization, knockdown by siRNA of GATAa	[51]
	Botryllus primigenus	In situ hybridization (Vasa, Nanos, Piwi) Immunohistochemistry (Vasa, Nanos) TEM, Knockdown by siRNA of Piwi and Nanos	[45–47]
	Botrylloides leachii	Live imaging and histology	[71, 74, 77]
		Transcriptome profiling	[73]
		In situ hybridization	[73, 74, 77]
		Serin protease inhibition	[73]
		Immunohistochemistry, Western blot, blood cells collection and Dil staining, RNAi, Citral and DEAB administration, Mitomycin C treatment	[74]
		Chemical inhibition of HDAC activity, measurement of nuclear acetyl-proteins-proliferating cell antigen (PCNA) staining	[77]
	Botrylloides violaceus	Microscopy and live imaging, histology, immunocytochemistry (Piwi, PCNA), Western blot (Piwi)	[72]
	Botrylloides diegensis	Gene expression (qPCR for Vasa, Piwi1, Piwi2, IA6, pou3, Cyclin B, Histone 3,Notch and Wnt pathways components), in situ hybridization (IA6, Pou3, h3), immunohistochemistry (IA6, Pou3, histone h3 phospho S10), isolation via FACS of IA6 positive cells and proliferating cells, limit dilution of sorted cells, injection in mitomycin C treated colonies of sorted cells, tracking of EdU labeled cells, Notch and Wnt signaling inhibition (DAPT and endo-IWR1)	[52]
Isolation of regressing zooids in the colony	*Botryllus schlosseri*	Immunohistochemistry (Vasa, Pl10, IAP28, pH 3), gene expression (qPCR for IAP genes, PI3K/Akt pathway), IAP genes chemical inhibition (GDC-0152 and Birinapant)	[53]
Isolation of bud fragments	*Botryllus schlosseri*	Histology	[54]
	Clavelina lepadiformis	In vivo observation and histological analysis	[55]
	Aplidium pellucidum	In vivo observations	[39]
	Perophora viridis	In vivo observations	[39, 40]

(continued)

Table 4 (continued)

Experimental procedure	Species	Methods	References
Esophagus isolation	*Clavelina lepadiformis*	In vivo observations and histological analysis	[21]
Stolon isolation	*Clavelina lepadiformis*	In vivo observations	[21, 38]
		Histology	[21]
	Perophora viridis	In vivo observations	[25, 39, 40]
		Histology	[25]
	Polyandrocarpa zorritensis	Histology	[56]
Partial regeneration			
Damaging of individuals	*Botryllus schlosseri*	In vivo observations	[27]
	Clavelina lepadiformis	In vivo observations and histological analysis	[21]
	Pycnoclavella neapolitana	In vivo observations	[59]
	Polyandrocarpa misakiensis	X-ray irradiation, histology, immunohistochemistry	[60]
		Inhibition of retinoic acid, detection of the activity of b-gal, knockdown by siRNA of retinoic acid receptors, in situ hybridization	[61]
Regeneration of the colonial circulatory system			
Removal of tunic, vessels, and ampullae	*Botryllus schlosseri*	In vivo observations	[62–65, 78]
		Histology	[65]
		Immunohistochemistry	[63, 65, 78]
		TEM	[62, 65]
		Knockdown by siRNA	[62]
		In situ hybridization and qPCR	[62, 63]

The regeneration of the *Ciona* siphons attracted the attention of many researchers for the simplicity of the technique required: a scalpel and some anesthetic [28–30]. A full regeneration of the siphons has also been described in *P. mytiligera*, *Styela plicata* and *Herdmania momus*, whereas, *Microcosmus exasperatus* show a scrubby siphon reconstitution, suggesting an unequal distribution of the regenerative abilities among solitary species [31].

Jeffery and collaborators [32, 33] reinvestigated in detail the regeneration of the oral siphon in *C. intestinalis* (formerly called *Ciona intestinalis* type B) [34]. They demonstrated that both short-distance and long-distance processes are involved in oral siphon regeneration, the former based on the presence, in the

remaining siphon stump, of a local pool of progenitor cells, the latter relying on the migration of progenitor cells from niches in the pharynx. Short-distance recruitment of progenitor cells is mainly involved in the formation of the orange-pigmented sensory organs (Fig. 3b, f) and is much more influenced by the depletion of the progenitor cell reservoir through repeated ablations [32]. When the siphon is amputated at its base, only long-distance recruitment of progenitor cells occurs [32].

A spectacular example of regeneration regards the two congeneric species *P. mytiligera* and *P. tenera* that can eject their viscera when subjected to stress conditions and rebuild them in less than 3 weeks [13]. Moreover, individuals of *P. mytiligera* can be separated in fragments by cutting along the longitudinal or transverse body axes. Each fragment then is able to regenerate completely the missing organs forming independent functional individuals [14].

As stated above, the methods used for studying regeneration in solitary ascidians were, in the past, quite simple: a scalpel or a razor blade to cut the animals or ablate the siphon(s), a dissection microscope to observe the anesthetized animals after the operation, eventually equipped with a camera lucida apparatus to record the regenerating steps in the recovering specimens (see the following "methodological approaches" section for a detailed historical overview on the methods used to induce regeneration). Today, the experimental procedure is not greatly different. The only relevant change is the introduction of in vivo imaging, which renders reporting much easier, and of electron microscopy analysis that allows detailed observations of the events at cellular levels. In addition, it must be stressed that, today, research on regeneration can exploit the abundance of biochemical and biomolecular toolkits offering the possibility to study in detail the events occurring during recovery. Table 2 reports the various approaches used for the study of regeneration and the attempt to elucidate the cells, genes, signaling pathways involved in the process.

3 Regeneration in Colonial Ascidians

The first studies on regeneration in colonial ascidians are those of Giard [35] in the second half of the nineteenth century, cited by [36]. Driesh [36], using *Clavelina lepadiformis*, observed that, dividing the animals in two, each fragment was able to regenerate the missing parts. *C. lepadiformis* was also used as a model organism for regeneration studies by Della Valle [37, 38]. In 1921, Huxley was one of the first authors to study regeneration in *Perophora viridis*, by recording the regenerative process after splitting the zooids in two [39]. The same author also investigated the regenerative capability of *Aplidium pellucidum* by isolating small colonial fragments. More detailed studies were carried out on the

regeneration processes of *P. viridis* by Deviney, in 1934 [25], and by Goldin, in 1948 [40].

In the second half of the twentieth century, new model organisms, such as *B. schlosseri* and *Botrylloides leachii,* became the main protagonists of regeneration studies in colonial ascidians. Even today these two organisms are widely used for regeneration studies. Over the years, studies have focused on three aspects (Table 3):

1. WBR
2. Partial regeneration
3. Circulatory system regeneration

3.1 WBR

WBR was studied in *B. schlosseri*, *B. leachii*, *Botrylloides violaceus* and *Botrylloides diegensis* following the surgical removal of all zooids and buds from a colony (Fig. 3g–j) or isolating small fragments of the colonial vasculature. This type of regeneration closely resembles vascular budding, a spontaneous formation of new buds from the vessels of the vascular system, first described in botryllid ascidians more than 200 years ago [41] and observed and described again by Giard [35], Bancroft [42], and Herdman [43]. This type of budding is constitutive in *Botryllus primigenus* [44], but it can also be induced through the isolation of small vascular fragments containing part of the colonial circulatory system [45–47]. In WBR, a bud, that eventually reconstitutes the whole colony, develops in the colonial vasculature from the aggregation of hemoblasts [5, 48–52]. Recently, Rosner and collaborators observed and studied an additional form of WBR in *B. schlosseri,* termed “budectomy induced WBR.” When, in a colony at takeover (the phase in which the adult zooids are being resorbed and replaced by their primary buds), all the buds are surgically removed leaving only old zooids undergoing resorption, new budlets can develop from the latter [53]. In this view, even the experiments performed on *B. schlosseri* colonies by Majone in 1977 can be considered as WBR [54]. In this case, new budlets develop from anterior bud fragments connected to the colonial vasculature. These new budlets eventually grow further to actively filter-feeding adults [54].

In species in which zooids are connected by stolons, such as *Polyandrocarpa zorritensis*, *C. lepadiformis* and *P. viridis,* WBR has been induced through the isolation of part of the stolon from the rest of the colony [21, 25, 37–39, 55, 56]. The success and timing of regeneration depend on the dimension of isolated stolon fragments [25, 38, 40]. Full recovery can require a period of time ranging from a few days, as in *P. zorritensis* [56], to several weeks, as in the case of small pieces of stolon of *C. lepadiformis* [37]. In *A. pellucidum* and *P. viridis*, Huxley [39] isolated small fragments of colonies or even of zooids, and observed what he called the “dedifferentiation” of the latter to undifferentiated structures from which new buds eventually

developed [39]. These processes were further studied by Deviney in 1934 [25] and Goldin in 1948 [40].

In *B. schlosseri,* it is also possible to observe the regeneration of bud residuals. When, in a colony, a single adult individual is left and its primary and secondary buds are removed, bud residuals can reverse the degeneration process and start regeneration. This phenomenon, described for the first time by Sabbadin in 1956 [57, 58], probably occurs for the absence of competition among buds, allowing the residuals to rescue development. In this case, a constitutive degeneration of budlets is reversed in a regenerative process induced by the new colony condition. This is an example in which it is difficult to mark the border between asexual reproduction and typical regeneration, and it stresses the high homeostatic capacity of colonial organisms to survive adverse conditions.

3.2 Partial Body Regeneration

Partial body regeneration can also be observed in colonial species. In *B. schlosseri* [27], *C. lepadiformis* [21], *Pycnoclavella neapolitana* [59] and *P. misakiensis* [60, 61], the regeneration of the missing parts of amputated buds have been studied (Table 3). In these cases, no regression and development of new budlets are observed.

3.3 Tunic and Colonial Circulatory System Regeneration

Colonial ascidians have also been studied for the ability to regenerate their tunic and the colonial circulatory system. In *B. schlosseri*, the full regeneration of the tunic and circulatory system occur in few hours when the peripheral matrix (i.e., the tunic and the enclosed portion of vasculature) is removed [62, 63] or days, when also marginal and radial vessels are ablated from the colony (Fig. 1k, l) [64, 65].

As in solitary species, in more than a century of studies the surgical procedures employed to induce whole body or partial regeneration in colonial ascidians are roughly unchanged. However, the development of imaging and molecular tools allowed, in the last decades, the detailed study of the kinetics of regeneration, and the investigation of the molecular pathways involved in many aspects of WBR, as stem cells maintenance and differentiation (Table 4).

4 Methodological Approaches to Induce Regeneration: An Historical Overview

4.1 Solitary Ascidians

The first reports on induction of regeneration in solitary ascidians comes from Loeb (1892, cited in [36]), who observed siphon regeneration in *Ciona*, and Schultze [16], who documented siphon and brain regeneration and studied the process both in vivo and at the histological level. Almost 15 years later, Hirschler [18] cut individuals of *Ciona* transversely and obliquely through the thorax and utilized camera lucida drawings to record the regeneration process. Unfortunately, we do not have any information on the

methodological approach used by these authors to induce regeneration. In 1930, Wermel published some methodological notes to study the regeneration of the oral siphon: he anesthetized animals of 4–7 cm in length (with MS222 or 10% chloral hydrate) and used fine dissection scissors to cut tissues [28].

Similar approaches for inducing regeneration were followed by later authors up to the more recently published reports, with some minor differences, such as the use of a scalpel to perform the ablations after the anesthetizing step [31].

Consecutive amputations of the oral siphon were used to study the recruitment of progenitor cells required for regeneration in *Ciona* [32].

In 1915, Sélys-Longchamps described the evisceration in individuals of *Polycarpa tenera* kept in aquaria [12], also induced in the congeneric species *P. mytiligera* by Shenkar and Gordon [13], whereas Bourchard-Mandrelle [66] observed gonad regeneration in *Ciona* after their removal through a small hole in the body wall.

In 1992, Bollner and collaborators [67] set up the methodological approach for inducing brain regeneration in *Ciona*, used also in subsequent works [68]: after anesthesia with MS222 (0.02%) they first cut the epidermis and the nerves anterior to the neural complex to expose the anterior neural gland (ciliated funnel), and then proceeded through the epidermis toward the posterior part of the neural complex that was finally separated from the pharyngeal basket and the posterior nerves.

Dahlberg et al. [69] obtained better results than traditional microdissection in the induction of brain regeneration in *Ciona* by anesthetizing animals in MS222 (0.4 g/L) or propylene phenoxetol (0.06%) in seawater for 15–30 min before ablation, dissection or live imaging. For the ablation, they used fine forceps and biopsy punch tools (2 and 3 mm diameter). Animals were placed in silicone-coated Petri dishes and the entire cerebral ganglion was removed (with the associated neural gland, its ciliated funnel and the dorsal tubercle), in a single action to minimize the trauma. A different method to produce a brain lesion and induce its regeneration in *S. plicata* was described in 2015 by Medina and collaborators [70]: it consisted on the systematic injection in the pharyngeal region of the neurotoxin 3-acetylpyridine (3-AP; 65 mg/kg body weight), diluted in sterilized artificial seawater. This compound is a niacinamide antagonist that inhibits ATP synthesis, resulting lethal to the high metabolic rate of neurons. At selected time points following injection, they anesthetized, killed, and dissected the animals to collect their brains, which were then processed for their analysis.

Some general methodological approaches to induce regeneration in solitary ascidian can be summarized as follow:

1. Anesthetization of specimens, leave the animals in:
   (a) MS222 (e.g., 0.4 g/L in [69]) or
   (b) 10% (w/v) chloral hydrate [28] or
   (c) 0.06% (v/v) propylene phenoxetol [69] or
   (d) menthol crystals (e.g., 0.4% (w/v) in [31]).
2. Removal of body parts:
   (a) For amputations (siphons, thorax, etc.)
      - Dissection scissors and/or [28].
      - Scalpels (see for example [31]).
      - Forceps and biopsy punch tools (2 and 3 mm diameter) [69].
      - Dissection on petri dishes (e.g., Sylgard®-coated) [69].
   (b) For chemical induction of brain degeneration.
      - Injection, in the pharyngeal region, of 3-acetylpyridine (3-AP; 65 mg/kg body weight) diluted in sterilized artificial seawater [70].
   (c) For evisceration.
      - Specimens kept alive in aquaria [12].
      - Gently squeezing [13].

4.2 Colonial Ascidians

Up to the half of the nineteenth century, publications reporting experiments on colonial ascidians did not provide details on the methodological approaches used to induce regeneration. Publications simply report that individuals or stolons were cut and observed in vivo. Sometimes, regenerating fragments could be labeled with vital stains such as neural red, whereas fixed specimens were labeled with carminium for whole mount analysis [21]. Below, we report the available information on the three main kinds of regeneration in colonial ascidians.

One of the first studies on partial regeneration in *B. schlosseri* was that of Sabbadin, in 1956 [57]. In his study, he removed all the budlets (budectomy) of a colony except one with thin tungsten needles and razor blades under a dissecting microscope. The only remaining budlet was then removed when, after 72 h, it became a bud. The atrophied buds which are normally resorbed restarted their development and, eventually, became adults. Since 1956, numerous studies used *B. schlosseri* as a model organism to study WBR in colonial ascidians. In 1975, Sabbadin and Zaniolo [50] removed all the zooids and buds from colonies, with needles and razor blades under a dissecting microscope, leaving only the peripheral colonial matrix, that is, the tunic and its vasculature. They observed that, in a few days, a vascular bud developed from aggregation of blood cells and generated a new zooid. The same method

was used by Rinkevich and collaborators [71] in *B. leachii*, Brown and collaborators [72] in *B. violaceus*, and Sunanaga and collaborators [45–47] in *B. primigenus*. Over the past 20 years, studies on regeneration using *B. schlosseri* and *B. leachii* have been implemented using molecular and biochemical techniques, studying in detail the involvement and function of some genes in regenerative processes of this two colonial ascidian [5, 53, 73–77].

Studies on partial regeneration in *B. schlosseri* were performed by Watkins [27], who damaged, with sharpened steel needles, the colonial buds and observed that about half of them regenerated the damaged part and reached maturity. The same method was used by Kaneko and collaborators [61] to induce partial regeneration in *P. misakiensis*.

Up to now, the regeneration of the vascular system was studied only in *B. schlosseri*. Zaniolo and Trentin [64] removed the entire colonial matrix (tunic, vessels and ampullae) around some zooids using a tungsten needle under a dissection microscope and observed the full regeneration of the peripheral vessels and tunic in 5 days. The same method was used in later studies [62, 63, 65, 78].

A general methodological approach to induce regeneration in colonial ascidian can be summarized as follow:

1. Collect swimming larvae and induce their metamorphosis by osmotic shock (30 s in diluted seawater 1% (w/v) of salinity) and let them to adhere on glass slide where they can grow [79].
2. Maintain animals in aerated aquaria filled with filtered seawater (FSW) at a 14:10 h light–dark regimen under constant (18–20 °C) temperature. Daily, feed them with unicellular algae (e.g., *Tetraselmis chuii*) and change water.
3. Keep colony-containing slides vertically, using plastic racks and clean them with a brush at each change of water.
4. Perform operations using thin tungsten needles and razor blades under a dissection microscope.
5. After surgery, keep the colonies in FSW in aerated aquaria as previously described.

For WBR

1. Remove all the zooids, buds, and budlets from the colony, leaving only the colonial matrix [50].

For partial body regeneration

1. Remove the parts of interest of the animal from the colonies [27, 61].

For circulatory system regeneration

1. Remove the colonial matrix after cutting the radial vessels and the test all around the zooids [64].

5 Concluding Remarks

As evidenced by the studies reported here, tunicates exhibit remarkable regenerative abilities, which have been studied from many points of view in over a century of researches. Besides their easy rearing and maintenance under laboratory conditions, the methods to induce regeneration are relatively simple and require few and cheap tools. Moreover, among chordates, only tunicates regenerate complete adult individuals from small tissue fragments. Despite these important characteristics, many molecular tools are still missing for this group of organisms. Transgenesis is one example: this technique would allow the in vivo study of gene expression during regeneration phases. However, it is still not developed especially for colonial ascidians and many solitary species as well. Today, the improvement of imaging techniques, and the broad application of some molecular biology tools, like RNA sequencing, can speed up the advancement of knowledge in the regenerative biology of ascidians. The studies summarized here will serve as a reference and a starting point for future researches aimed to uncover the biological basic properties of the astonishing regeneration in these chordates.

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