Current Knowledge on Stem Cells in Ascidians

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Abstract: Ascidians belong to tunicates, the sister group of vertebrates. Ascidians are cosmopolitan marine filter-feeding organisms that, along with other members of the chordate subphylum, maintain remarkable regenerative abilities throughout their life. Ascidians' high stem-cell-mediated regenerative capacity, which allows colonial species to continuously generate new individuals, has fascinated researchers and scientists. In this chapter, we emphasize what is currently known about the biology and level of involvement of stem cells in ascidian development and regeneration for both solitary and colonial species. The chapter focuses on the methods used to identify stem cells and stem cell niches and discusses hypotheses regarding their role in biological phenomena such as budding, torpor, regeneration, aging, and chimerism. Future areas of study on stem cells using regenerative ascidians are discussed.

1. Introduction

Tunicates, the sister group of vertebrates (Figure 1) (Delsuc et al. 2006, 2018), are filter-feeding marine invertebrates found in harbors, estuaries, and oceans around the world (Burighel and Cloney 1997; Holland 2016).

As members of the phylum Chordata, tunicates develop from swimming larvae that contain all the primary chordate features such as a notochord, dorsal neural tube, segmented musculature, and gill slits (Brusca et al. 2016). After a swimming phase, the larva loses many of its chordate characteristics, metamorphosing into a sessile or pelagic individual (Brusca et al. 2016). Ascidians are the group most studied among tunicates and include both solitary (Figure 1b–e) and colonial (Figure 1f–l) species. Solitary ascidians reproduce via embryogenesis, with individuals developing from a single fertilized egg, while the colonial species produce an adult body through both embryogenesis and diverse types of asexual reproduction (Brien and Brien-Gavage 1928; Oka and Watanabe 1957a; Freeman 1964; Sköld et al. 2011; Lemaire 2011; Manni et al. 2019; Kowarsky et al. 2021). These two disparate reproductive methods ultimately give rise to a similar adult body plan consisting of a simple central nervous system, digestive system, respiratory system, circulatory system, and reproductive system (Manni et al. 2019; Kowarsky et al. 2021) (Figure 1a–d).

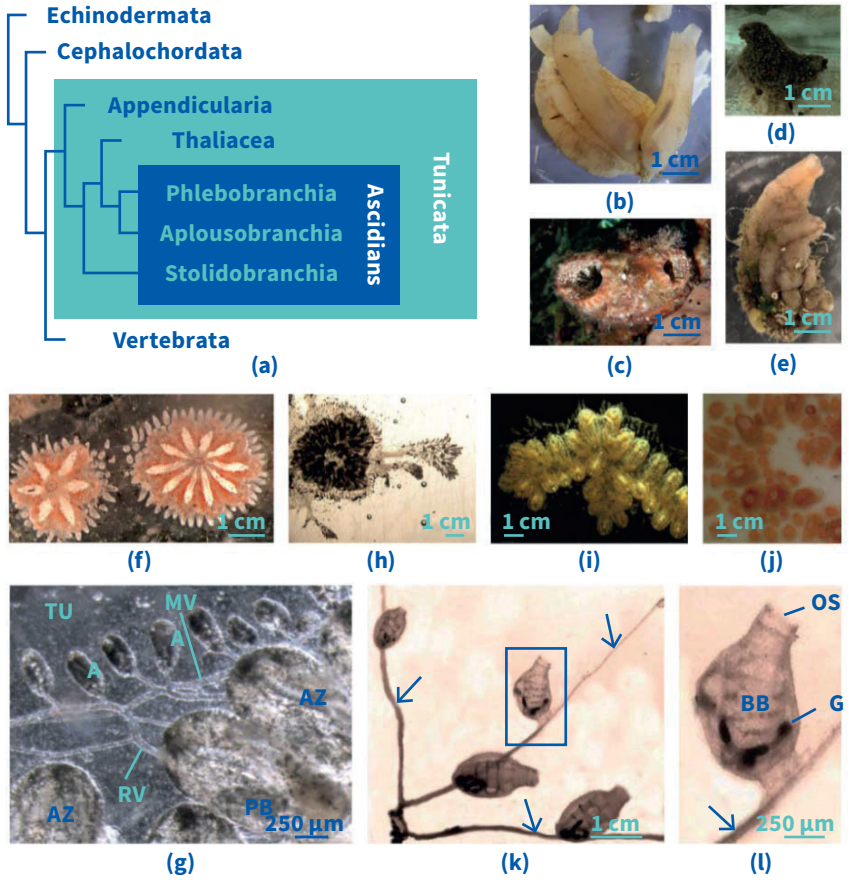

Figure 1. (**a**) Phylogenetic tree of deuterostomes (modified from (Delsuc et al. 2018). Ascidians are considered a paraphyletic group. The three taxa—namely, Phlebobranchia, Aplousobranchia, and Stolidobranchia, also include colonial species able of budding. (**b–e**) Solitary ascidians: *Ciona robusta*, lateral view (**b**); *Polycarpa mytiligera*, upper view (**c**); *Microcosmus exasperatus*, upper-lateral view (**d**); *Styela plicata*, lateral view (**e**). (**f–l**) Colonial ascidians (dorsal view): *Botryllus schlosseri* (**f**) and detail of the colonial circulatory system (**g**, ventral view); *Botryllus primigenus* (**h**); *Botrylloides leachii* (**i**); *Polyandrocarpa zorritensis* (**j**); *Perophora viridis* (**k**,**l**); arrowheads in (**k**,**l**): stolon. The Square area in (**k**) is enlarged in (**l**). A: ampulla; AZ: adult zooid; BB: branchial basket; G: gut; MV: marginal vessel; OS: oral siphon; PB: primary bud; RV: radial vessel; TU: tunic. Source: Graphic by authors.

Both solitary and colonial species have high regenerative capacities, with colonial species regenerating an entire body plan from a small fragment of its vasculature (Oka and Watanabe 1957a; Sabbadin et al. 1975; Rinkevich et al. 2007a, 2007b, 2008; Voskoboynik et al. 2007; Manni et al. 2014, 2019; Alié et al. 2021). When colonies

come in contact with each other, colonies may form natural chimeras with adjacent colonies by vascular fusion if they share one or two alleles in their highly polymorphic histocompatibility gene, the *Botryllus* histocompatibility factor (BHF); if the colonies are incompatible, a barrier forms between them, and they reject (Oka and Watanabe 1957b; Sabbadin 1962; Scofield et al. 1982; Voskoboynik et al. 2013b).

Following fusion, the circulating stem cells of the chimeric partners compete to replace the germline and/or the soma of the other partner in a process similar to allogeneic transplantation (Oka and Watanabe 1957b, 1959; Sabbadin and Zaniolo 1979; Pancer et al. 1995; Stoner and Weissman 1996; Stoner et al. 1999; Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013).

The colonial ascidians' abilities to reproduce sexually and asexually, regenerate whole body plans, and replace the genotypes of germline and somatic tissues in chimeras has prompted studies aiming to identify and prospectively isolate the stem cells involved in these events.

In this chapter, we review the current knowledge on the stem and progenitor cells in solitary and colonial ascidians, and their involvement in developmental/regeneration processes. Special emphasis is given to the methods used to identify/isolate candidate stem cells and their niches.

2. Ascidians as Model Organisms for Developmental Studies

At the end of the 18th century, studies performed on ascidians established them as key models of chordate development (Corbo et al. 2001; Satoh 2001; Lemaire 2011; Stolfi and Christiaen 2012), sexual and asexual reproduction (Manni et al. 2019; Kowarsky et al. 2021), and the evolution of the immune system (Scofield et al. 1982; Cooper et al. 1992; Oren et al. 2013; Voskoboynik et al. 2013b; Ballarin et al. 2015, 2021a; Franchi et al. 2017; Rosental et al. 2018; Mueller and Rinkevich 2020).

Through a classic chordate embryogenesis process, ascidians produce swimming tadpole-like larvae that, following metamorphosis, lose their chordate characteristics (Lemaire et al. 2008). Taking advantage of the transparent embryos of solitary ascidians, Conklin (1905) performed the first cell lineage experiment in *Styela partita* embryos and discovered that, at the cleavage stage, cells (blastomeres) are committed to the three germ layers: ectoderm, mesoderm, and endoderm. Conklin's studies established ascidians as a key model for embryogenesis. Today's advanced transgenic lineage tracing techniques and single-cell transcriptome trajectories are used on ascidian *Ciona* species to build comprehensive embryonic cell fate maps (Dehal et al. 2002; Lemaire 2011; Oonuma et al. 2016; Tolkin and Christiaen 2016; Cao et al. 2019; Lemaire et al. 2021).

The most studied solitary ascidians for developmental research are the widely distributed *Ciona robusta* (Figure 1b) and *Ciona intestinalis*. The genome of *C. robusta* was one of the first genomes assembled (Dehal et al. 2002), allowing molecular studies

on the origin of chordates. Embryos are obtained by in vitro fertilization, and gene reporter assays are used to monitor and manipulate gene expression in vivo as the embryo develops (Squarzoni et al. 2011; Stolfi and Christiaen 2012; Racioppi et al. 2014; Farley et al. 2015; Fujiwara and Cañestro 2018).

Among colonial ascidians, *Botryllus schlosseri* is one of the reference colonial species. Several features make *B. schlosseri* an excellent model organism (Figure 1f,g)—namely, (i) it is abundant in shallow waters and easily cultured in the laboratory; (ii) its genome and transcriptome are available (Voskoboynik et al. 2013a, 2013b; Corey et al. 2016; Campagna et al. 2016; Rosental et al. 2018; Kowarsky et al. 2021; Voskoboynik et al. 2020; Anselmi et al. 2021); (iii) asexual reproduction results in identical individuals, facilitating the ability to separate one colony (genotype) into several clonal replicates (Manni et al. 2007, 2014; Kowarsky et al. 2021); (iv) it naturally forms chimeras, which allow lineage tracing by DNA fingerprints (Stoner and Weissman 1996; Laird et al. 2005); (v) its transparent tissue allows in vivo tracing of labeled cells (Voskoboynik et al. 2008; Rinkevich et al. 2013; Rosental et al. 2018).

3. Stem Cells and Their Identification

The term stem cell derives in part from the word Stammzelle, first used by Ernst Haeckel in the mid-1800s to describe both the single-celled organism precursors to multicellular life and the single-celled embryo that develops into a multicellular organism. The term and its concept were later used by August Weissman to describe cells that he hypothesized to be the common precursor of a specific tissue.

Stem cells must satisfy the following criteria to be classified as stem cells: (i) they can divide and create an identical copy of themselves (self-renewal), and (ii) they can divide to produce other cell types (e.g., hematopoietic stem cells (HSCs) produce all blood cells). They can also express a specific gene signature (e.g., piwi, vasa) and demonstrate a high nucleus:cytoplasm ratio.

Studies on the proliferation state of mammalian HSCs demonstrate that HSCs are quiescent most of the time (remaining in G0) and only on rare occasions enter the cell cycle (Passegué et al. 2005; Forsberg et al. 2010); therefore, proliferation markers, including EdU and PCNA, that detect proliferating progenitor cell populations in many cases do not identify stem cells.

To isolate a pure population of self-renewing HSCs, the Weissman group (Spangrude et al. 1988; Uchida and Weissman 1992; Morrison and Weissman 1994) developed methods that used (i) FACS-based monoclonal antibody cell separation technologies to isolate specific cell populations; (ii) transplantation of limited dilutions of these cell populations to irradiated mice and long term tracing of transplanted cells to assay multipotentiality; (iii) reisolation and transplantation of candidate stem cells from primary recipients to secondary hosts and long term tracing of transplanted cells to assay self-renewal. These became the standard methods to isolate adult

tissue-specific stem cells and were used to isolate various tissue-specific stem cells including neural (Uchida et al. 2000) and skeletal stem cells (Chan et al. 2018). A genetic approach that uses fluorescent reporter genes to trace differentiation of single cells was developed to isolate the gut stem cells (Barker et al. 2007). This genetic tracing method also reveals self-renewal and multipotency characteristics.

In order to confirm the involvement of candidate stem cells in regeneration in ascidians, cellular and transgenic methods such as the development of ascidian specific monoclonal antibodies, FACS protocols, transplantation protocols, transgenic animals, lineage tracing, and in vivo cell tracking are required.

4. Stem Cells in Ascidians

Observing *C. intestinalis* hemolymph, Rowley (1982) used the term to indicate cells with a high nucleus:cytoplasm ratio typical of undifferentiated cells. Kawamura et al. (1991) used the same term for cells with similar morphology that migrate and aggregate in the developing buds of *Polyandrocarpa misakiensis*.

The vast majority of data on stem cells in ascidians emerged from studies on *Botryllus schlosseri*. Observing genotype replacement of germline and somatic tissues in *B. schlosseri* chimeras led Pancer et al. (1995) and Stoner and Weissman (1996) to hypothesize that *B. schlosseri* chimerism, cell parasitism, and budding are mediated by stem cells (Pancer et al. 1995; Stoner and Weissman 1996; Stoner et al. 1999; Rinkevich and Yankelevich 2004).

The ability of allogeneic *B. schlosseri* colonies to form chimeras if they share one or two alleles in their histocompatibility gene BHF (Voskoboynik et al. 2013b) allows lineage tracing of transplanted cells using allele-specific markers of host and donor as genotype barcodes (Figure 2) (Pancer et al. 1995; Stoner and Weissman 1996; Stoner et al. 1999; Rinkevich and Yankelevich 2004; Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). By microinjecting $2.5–5 \times 10^4$ hemocytes into allogeneic partners, Pancer et al. (1995) documented co-sharing and even replacement of the gonads in the recipient partners by the donor cells, as well as continuous somatic chimerism. By transplanting a small number of cells that expressed high aldehyde dehydrogenase activity (ALDH, a stem cell marker), and a set of serial engraftment assays (Figure 2a,b), Laird et al. (2005) further proved that in *B. schlosseri* stem cells are mediating both chimerism and budding. Using transplantation experiments, in vivo cell labeling, and tracing, the anterior ventral side of the endostyle and the cell islands were identified as niches for somatic and germline stem cells (Voskoboynik et al. 2008; Rinkevich et al. 2013). Cells from the endostyle niche migrated via the branchial sac sinuses to buds and contributed to their development (Voskoboynik et al. 2008). Cells from the cell islands migrated to the developing gonads and contributed to their development (Rinkevich et al. 2013). Rosental et al. (2018) adapted FACS to characterize *B. schlosseri* circulating cells and isolated 24 populations. Transcriptome

analysis of these populations revealed a cluster of 3 cell populations that differentially upregulated 235 genes homologous (based on sequence) to mammalian genes, known to be expressed in the mammalian hematopoietic stem, progenitor, and myeloid lineage cells. It also revealed three cell populations that highly expressed genes homologous to mammalian genes expressed in cells and tissues of the human reproductive system (testes, ovary, placenta, sperm, and germline). Transplantation experiments and lineage tracing further demonstrated the multipotent potential of the cHSC populations (Rosental et al. 2018) (Figure 2d). Transplantation of labeled cells and in vivo tracing experiments demonstrated migration of cHSC to the endostyle niche, while the cGSC identified based on sequence migrated to the cell island niche (Rosental et al. 2018).

In both colonial and solitary ascidians, it was suggested that wound response and tissue regeneration are mediated by stem cells (Voskoboynik et al. 2007; Voskoboynik and Weissman 2015; Blanchoud et al. 2018; Jeffery 2019; Kassmer et al. 2020; Qarri et al. 2020). A population of circulatory cells was proposed to have stem cell potency (Kawamura et al. 1991; Stoner and Weissman 1996; Voskoboynik et al. 2007; Tiozzo et al. 2008a; Brown et al. 2009; Jeffery 2015a; Kassmer et al. 2020). Genotyping of somatic and germline tissues several months after transplantation of a few cells isolated from specific niches (e.g., endostyle niche, cell islands) or expression specific markers (ALDH) (Figure 2a; Table 1) demonstrated the ability of these cells to contribute to somatic or germline organs (Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). Transplantation of single cells with high ALDH expression and lineage tracing of their contribution to germline or somatic *B. schlosseri* tissues revealed contribution to either soma (buds) or germline (tests) but not both (Laird et al. 2005) (Figure 2a,b). These results strongly suggest that *B. schlosseri* stem cells are not pluripotent, i.e., they do not produce both germline and soma. The identification of candidate hematopoietic stem cell and germline cell populations in the colonial ascidian *B. schlosseri* (Rosental et al. 2018) (Figure 2c,d) suggests that tissue-specific stem cells mediate bud formation in colonial ascidians. Tissue-specific transcriptional signature and organogenesis timeline during embryogenesis and blastogenesis also strongly support this notion (Kowarsky et al. 2021).

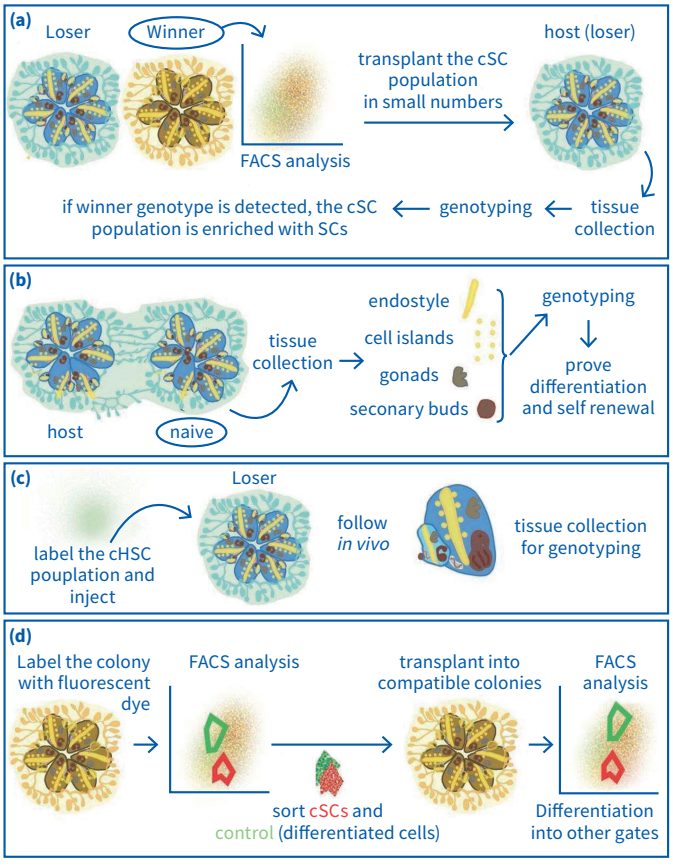

Figure 2. Assaying multilineage contribution, self-renewal capacities, and homing sites of *B. schlosseri* prospective isolated stem cells: (**a**) transplantation of candidate stem cell populations between genetically distinct but compatible colonies and use of tissue genotyping to determine the full developmental potential of transplanted cells; (**b**) primary recipients are fused with secondary naive hosts several months following initial transplantation, tissue genotyping of secondary hosts is used to assay self-renewal capacities; (**c**) candidate stem cells are isolated by FACS, labeled with fluorescent dyes, and transplanted to *Botryllus* blood vessels. Cells are traced in vivo using confocal microscopy via the transparent body of the colonies to identify the location of stem cell niches; (**d**) FACS-based analysis used to demonstrate candidate stem cells differentiation ability. Candidate stem cell and control populations are labeled with fluorescent dyes and transplanted into compatible hosts, a few weeks following transplantation the fluorescent cells from the recipient colonies are analyzed by FACS. While the majority of the transplanted control cell populations are expected to remain in their original gate, the majority of the transplanted candidate stem cell populations are expected to be detected in gates different from their original gates (suggesting they were differentiated). Source: Graphic by authors.

Table 1. Genes expressed in ascidian candidate stem and progenitor cells.

Gene	Species	Expressing Cell(S)	Methods	References
RNA-binding proteins				
Argonaute family silencing genes				
piwi	*Botryllus schlosseri* *Botrylloides leachii* *Botrylloides violaceus* *Botrylloides diegensis* *Ciona intestinalis* *Styela plicata*	hemoblasts, phagocytes, tunic cells, stomach cells, cell islands, endostyle, epithelial cells of the vasculature	ISH, IHC; iRNA	(Brown et al. 2009; Rosner et al. 2009; Rinkevich et al. 2010, 2013; Jeffery 2015d; Jiménez-Merino et al. 2019; Kassmer et al. 2020)
DEAD and DEAH-box-containing helicases				
vasa	*Botryllus schlosseri* *Botrylloides violaceus* *Botrylloides diegensis*	hemoblasts, epithelial cells, phagocytes, stomach cells, cell islands	ISH, IHC	(Brown and Swalla 2007; Rosner et al. 2009; Rinkevich et al. 2013; Kassmer et al. 2020)
pl10	*Botryllus schlosseri*	epithelial cells, some blood cells, phagocytes in cell islands, stomach cells	ISH, IHC, ICC	(Rosner et al. 2006, 2009)
ddx1	*Botryllus schlosseri*	cell islands	IHC, ISH	(Rosner et al. 2013)
Nanos family proteins				
nanos	*Botryllus primigenus*	pharyngeal epithelia of developing budlets	ISH, IHC	(Sunanaga et al. 2008)
RNA recognition motif (RRM)-containing proteins				
dazap1	*Botryllus schlosseri*	buds, during blastogenesis, cHSC	ISH; RNAseq	(Gasparini et al. 2011; Rosental et al. 2018)
Signal transduction pathways				
Wnt				
wnt2B, *wnt5a*, *wnt7a*, *nt5A*, *wnt9Aβ-cat*	*Botryllus schlosseri*	secondary buds (stages 1-3), developing gonads, primary buds, cHSC, endostyle	ISH, RNAseq	(Rinkevich et al. 2013; di Maio et al. 2015; Rosental et al. 2018)
fzd5/8, *β-cat*, *dsh*	*Botrylloides diegensis*	cycling hemoblasts	ISH	(Kassmer et al. 2020)
TGF-β/BMP				
smad1/2/5/8	*Botryllus schlosseri*	phagocytes, endostyle, cHSC	IHC, ISH, RNAseq	(Rosner et al. 2013; Rosental et al. 2018)

Table 1. *Cont.*

Gene	Species	Expressing Cell(S)	Methods	References
		Notch		
notch1, notch2, notch3, hes1	*Botrylloides diegensis, Botryllus schlosseri*	cycling hemoblasts during WBR, endostyle, cHSC	ISH, RNAseq	(Rosental et al. 2018; Kassmer et al. 2020)
		Kinases		
pm-rack1	*Polyandrocarpa misakiensis*	atrial epithelium of developing buds, undifferentiated mesenchymal cells, pharynx epithelium	ISH, IHC	(Tatzuke et al. 2012)
		Homeobox-containing proteins		
pitx, oct-4, pou-3	*Botryllus schlosseri*	budlets at stage 1-3, oral siphon and tentacles; forming cerebral ganglion, endostyle, developing gut, epithelial cells	ISH, IHC, qRT-PCR	(Tiozzo et al. 2005, 2009; Rosner et al. 2009; Tatzuke et al. 2012; Rinkevich et al. 2013; Ricci et al. 2016)
pou3	*Botrylloides diegensis*	hemoblasts	ISH	(Kassmer et al. 2020)
		Zinc-finger proteins		
GATA4/5/6	*Botryllus schlosseri*	atrial epithelium of the bud at stage 3	ISH	(Ricci et al. 2016)
myc	*Botryllus primigenus, Polyandrocarpa misakiensis*	branchial epithelia, circulating hemocytes of growing palleal and vascular buds (Bs); cells of the atrial epithelium and fibroblast-like cells involved in organogenesis (Pm)	ISH	(Sunanaga et al. 2008; Fujiwara et al. 2011; Kawamura and Sunanaga 2011)
		Chromatin modification/cell cycle/differentiation		
		Histones		
p-h3	*Botryllus schlosseri, Botrylloides diegensis, Styela plicata*	primary and secondary buds, zooidal stomach (Bs, Bd) hemoblasts, intestine submucosa, adults (Sp)	IHC, ISH	(Rosner et al. 2014; Jiménez-Merino et al. 2019; Kassmer et al. 2020)

Table 1. *Cont.*

Gene	Species	Expressing Cell(S)	Methods	References
		Proliferation markers		
pcna	*Polyandrocarpa misakiensis*, *Botrylloides violaceus*, *Botryllus schlosseri*	cells of the atrial epithelium of the developing buds, during WBR, hemocytes, cHSC	IHC, RNAseq	(Brown et al. 2009; Kawamura et al. 2012; Rosental et al. 2018)
cyclin b	*Botrylloides diegensis*	hemoblasts during WBR	ISH	(Kassmer et al. 2020)
		Cytostatic proteins		
tc14-1, *tc14-3*	*Polyandrocarpa misakiensis*	atrial epithelial cells of growing buds, hemoblasts	IHC	(Kawamura et al. 1991; Matsumoto et al. 2001)
		Telomere protection		
pot1	*Botryllus schlosseri*	multipotent epithelia of budlets	ISH	(Ricci et al. 2016)
		Proteins involved in autophagy		
Pm-atg7	*Polyandrocarpa misakiensis*	atrial epithelium of developing buds	ISH	(Kawamura et al. 2018)
		Control of differentiation		
raldh	*Botrylloides leachii*, *Botryllus schlosseri*	circulating phagocytes, inner epithelium of the bud, endostyle	ISH	(Rinkevich et al. 2007b, 2013; Ricci et al. 2016)
if-b	*Botrylloides leachii*	atrial epithelium of buds	ISH	(Ricci et al. 2016)
		Niche interaction		
cadherin	*Botryllus schlosseri*	aggregates of hemoblasts, aggregates of phagocytes near the endostyle, bud epithelia	ISH, IHC	(Rosner et al. 2007)
cd133	*Botryllus schlosseri*	ampullae epithelium during vasculature regeneration, some hemocytes	ISH, FACS	(Braden et al. 2014)
Ia-6	*Botrylloides diegensis*	hemoblasts	ISH	(Kassmer et al. 2020)
		Others		
Pm-pumpA	*Polyandrocarpa misakiensis*	atrial epithelium of developing buds	ISH	(Kawamura et al. 2018)

Table 1. *Cont.*

Gene	Species	Expressing Cell(S)	Methods	References
Pathways associated with stem cell activity and stem cell niches				
Wnt signaling, Signaling by *Notch*, *SMAD* signaling (See Figure 4g for more)	*Ciona*	endostyle	ISH	(Ogasawara et al. 2002)
Wnt/ RET/ HIF-1/ VEGF/ signaling, *Nanog* ESC pluripotency (See Figure 6e for more)	*Botryllus schlosseri*	endostyle cHSC	RNAseq	(Rosental et al. 2018)

5. Development and Regeneration in Ascidians

As stated above, ascidians reproduce through two different pathways—either classic embryogenesis or blastogenesis where an adult organism develops via budding. Solitary ascidian species are restricted to the sexual mode of reproduction, while colonial species reproduce both ways.

The ability to replace or restore cells, tissues, and organs in response to either damage or loss is a remarkable regenerative function shared by many organisms. Ascidians vary in their regeneration capacities from those that have a limited regeneration to those that can replace any missing body part or even regenerate a complete organism.

5.1. Development, Regeneration, and Stem Cells in Solitary Ascidians

5.1.1. Embryonic Development

In solitary ascidians, fertilization occurs externally, and embryos develop on the water's column; a vacuolated layer of internal follicular cells can keep the embryos floating. During embryogenesis, pluripotent embryonic cells gradually restrict their developmental potential as they become committed toward particular tissues or cells. By correlating single-cell transcriptomic data with knowledge regarding cell lineages, recent works systematically examine lineage specification during development in solitary species (Kobayashi et al. 2013; Cao et al. 2019; Ilsley et al. 2020; Sladitschek et al. 2020; Zhang et al. 2020). These studies reveal asymmetric cell divisions and conserved

expression of transcription factors involved in cell differentiation trajectories between ascidians and mice and may lead to the identification of the precursors of somatic or germ stem cells in adults. Additional information on germ stem cells is shown below.

5.1.2. Regeneration in Solitary Ascidians

Solitary ascidian reproduction is strictly sexual, and their regeneration capacity has been investigated in a few chosen species since the 19th century (reviewed in Jeffery 2015b). The majority of these species can only regenerate specific body parts, such as the siphons and neural complex (i.e., the brain and the associated neural gland) (Table 1).

The first report on regeneration in *Ciona* dates back to 1891, when Mingazzini, at the Stazione Zoologica in Naples (Italy), demonstrated that the oral and atrial siphons, as well as the brain, could regenerate following ablation (Mingazzini 1891). Later, Hirschler (1914) discovered *Ciona*'s basal body portion can regenerate distal organs, even though the distal portion cannot similarly regenerate basal organs such as the digestive system and heart. The basal body part is able to regenerate distal organs within one month as long as a fragment of branchial sac remains in the basal portion of the body (Jeffery 2015c). During the last century, new studies have confirmed these results and further described the cellular and molecular processes underlying tissue regeneration in this model system (Jeffery 2015a, 2015b, 2015c, 2019).

Partial body regeneration was also studied in the solitary ascidian *Styela plicata* (Stolidobranchia) (Table 2). This species can regenerate both the oral and atrial siphon following ablation (Gordon et al. 2019). By using the niacinamide antagonist 3-acetylpyridine (3AP) that causes lesions in the brain and reduction of glial and neuronal cells, Medina et al. (2015) demonstrated neuron regeneration in this species and the recruitment of circulating candidate stem cells to the lesion site.

A comparative study on the regenerative abilities of four solitary stolidobranch ascidians, *Polycarpa mytiligera*, *Herdmania momus*, *Microcosmus exasperatus*, and *S. plicata* (Figure 3) reported variation in regeneration potential among these species (Gordon et al. 2019). While all species survived and initiated regeneration following ablation of their siphons, only *P. mytiligera* survived the ablation of a larger portion of its body, including both siphons and the brain. A recent study further examined *P. mytiligera's* regenerative capacity (Gordon et al. 2021). In this study, individuals were cut in two or three fragments along the longitudinal and transverse body axis. After a month, each fragment had reconstituted the whole body and was physiologically active, able to filter feed and respond to stimuli (Figure 3a–c). *P. mytiligera*'s ability to regenerate all tissue and organs distinguishes it from the other solitary species studied so far (Gordon et al. 2021), emphasizing the wide range of regenerative abilities among closely related species. Comparative studies of these species will

shed light on the mechanisms underlying regeneration and the evolution of this complex process.

Table 2. Solitary ascidians regeneration capacities and the source of candidate stem cells that mediate it. N/A: not available.

Species	Regenerative Body Structures	Candidate Stem Cell Source	Methods Used to Identify Candidate Stem Cells	References
Ciona intestinalis Ciona robusta	Siphons, neural complex, and branchial basket	Branchial basket	Proliferation (EdU, Notch signaling) and stemness markers (PIWI, alkaline phosphatase), Transplantation experiments	(Dahlberg et al. 2009; Auger et al. 2010; Jeffery 2015a, 2015b, 2015c, 2019; Hamada et al. 2015)
Polycarpa mytiligera	Siphons, neural complex, branchial basket, digestive system, and heart	N/A	N/A	(Shenkar and Gordon 2015; Gordon et al. 2019, 2021)
Styela plicata	Siphons and neural complex	Intestinal submucosa, Branchial basket	Morphological characterization proliferation (pHH3) and stemness markers (Aldehyde dehydrogenase activity, PIWI, CD34)	(Medina et al. 2015; Gordon et al. 2019; Jiménez-Merino et al. 2019)
Microcosmus exasperatus	Siphons	N/A	N/A	(Gordon et al. 2019)
Herdmania momus	Siphons	N/A	N/A	(Gordon et al. 2019)

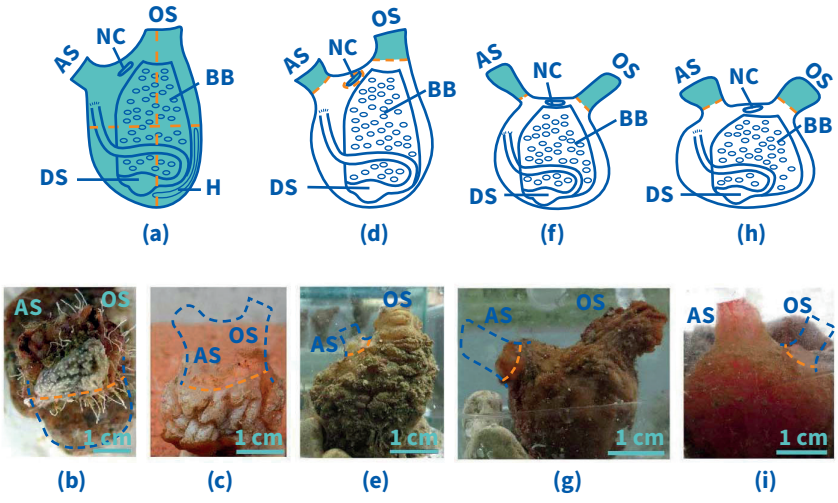

Figure 3. Solitary ascidians regeneration capacity: (**a**–**c**) *Polycarpa mytiligera*: (**a**) illustration summarizing body structures and regeneration capabilities. Dashed red lines indicate ablation lines. Regenerative body parts are highlighted in purple. Note that all organs can be regenerated following amputations; (**b**,**c**) in vivo images of regenerated animals 30 days following ablation along the anterior–posterior body axes: (**b**) anterior body part following ablation of posterior structures (indicated by a black dashed line). Note the open oral (OS) and atrial (AS) siphons; (**c**) posterior body part following ablation of anterior structures. Note the regenerated oral and atrial siphons. (**d**,**e**) *Styela plicata*: (**d**) illustration summarizing body structures and regeneration capabilities; (**e**) in vivo image of atrial siphon regeneration 30 days following ablation. Note the regenerated atrial siphon. (**f**,**g**) *Microcosmus exasperates*: (**f**) illustration summarizing body structures and regeneration capabilities; (**g**) in vivo image of atrial siphon regeneration 30 days following ablation. Note the regenerated atrial siphon. (**h**,**i**) *Herdmania momus*: (**h**) illustration summarizing body structures and regeneration capabilities; (**i**) in vivo image of atrial siphon regeneration 30 days following ablation. Note the regenerated oral siphon. BB: branchial basket; DG: digestive system; H: heart; NC: neural complex. Source: Graphic by authors.

5.1.3. Regeneration in *Ciona*

The involvement of candidate stem cells in solitary ascidian regeneration in response to injury is suggested by morphological, proliferation, and cell migration studies, as well as stem-cell-associated gene expression (Ermak 1975; Jeffery 2019; Jiménez-Merino et al. 2019; Kassmer et al. 2019). However, it is still unclear whether regeneration is accomplished by the proliferation of differentiated cells, activation

of quiescent stem cells, recruitment of progenitor cells, or a combination of these strategies.

As stated above, *C. robusta* and *C. intestinalis* are the main solitary species used to study regeneration (Bollner et al. 1992; Dahlberg et al. 2009; Auger et al. 2010; Jeffery 2015a, 2015b, 2015c, 2019). Members of the genus *Ciona* are among the most abundant invasive marine species with a wide geographic distribution (Lambert and Lambert 1998; Lambert 2001; Madariaga et al. 2014). The high accessibility of the species, combined with a simple, transparent body structure and a relatively short life span, associated with the availability of a sequenced genome and various transcriptomes, render these organisms useful models for experimental studies on regeneration (Millar 1952; Jeffery 2015c; Satoh 2019).

Early studies suggested a possible role for circulatory hemoblasts in regeneration (Hirschler 1914; Sutton 1953) (Figure 4). Using histological and light microscopy, these pioneering researchers described tissue regeneration in detail. Following these reports, *Ciona* regeneration research remained relatively silent, until a renewed interest in the field arose with the emergence of stem cell research in the 1990s. Recent studies introduced advanced molecular tools, such as in situ hybridization, immunofluorescent staining, and gene expression to further analyze the cellular and molecular process underlying *Ciona* regeneration. They focused, in particular, on the possible role of adult stem cells in wound response and regeneration (Hamada et al. 2015; Spina et al. 2017; Jeffery 2019; Kassmer et al. 2019; Jeffery and Gorički 2021).

Most regeneration studies on *Ciona* focused on the ability of this species to regrow its oral siphon (OS) (Figure 4c). The OS is composed of longitudinal and circular muscle fibers entrapped within a dense extracellular matrix where vascular sinuses and nerve fibers are present. Externally, the epidermis and the tunic layer cover it. At the base of the OS, a ring of tentacles embeds the coronal organ, a mechanosensory structure (Manni et al. 2006). Ciliated receptor cells at the center of a cup-like structure of orange pigmented cells form the eight pigmented oral siphon sensory organs (OPOs) located along the rim of the OS. The brain, from which several nerves originate, lies at the OS base (Dilly and Wolken 1972; Auger et al. 2010).

Following amputation, the OS regeneration proceeds through the following three phases: (i) formation of wound epidermis, (ii) OPO replacement, and (iii) OS regrowth (Auger et al. 2010). Full siphon regeneration requires a blastema formation supported by the migration of proliferating cells from the branchial sac (long-distance regeneration). However, the regeneration of siphon tip (including OPOs) most likely involves the differentiation of candidate quiescent stem cells already present in the siphonal tissues (short-distance regeneration) (Auger et al. 2010; Jeffery 2015b). The position of the amputation line controls the extent to which short- and long-distance regeneration processes are used (Figure 4c): removal of the entire OS leads to a complex regeneration process that involves both local cells and cells migrating from

the lymph nodules of the branchial sac. Conversely, when the amputation line is close to the siphon tip, it results in a faster regeneration process, relying only on local cell reservoirs (Jeffery 2015c). The latter assumption results from the observation that when the OS is fully removed from its base, the patterning of OPO regeneration is not fully conserved, showing duplications in the OPO number. Conversely, the removal of the distal part of the OS results in the complete replacement of the OPOs, both in numbers and structure, even after repeated amputations (Auger et al. 2010). In addition, UV irradiation of the siphon blocks OPO replacement following the removal of the siphon tip, supporting the idea that regeneration is mediated by local events (Auger et al. 2010). The regenerative capacities of *Ciona* are related to age and are compromised in older animals; when siphons are amputated in old animals at any position, the regeneration is often delayed or absent (Jeffery 2015a, 2015d).

Gene expression analysis of regenerating structures in *C. intestinalis* shows upregulation of conserved regulatory signaling pathways such as Notch and TGF-beta, as well as apoptosis-related genes (Hamada et al. 2015; Spina et al. 2017; Jeffery and Gorički 2021) (Figure 4g). Consequently, when the Notch pathway is inhibited, the levels of cell proliferation in the *Ciona* branchial sac and OS blastema is reduced (Hamada et al. 2015), and differentiation of OS muscle cells in the regenerating area is also affected. In particular, recent data indicate that apoptosis is required for OS regeneration and branchial sac homeostasis through activation of Wnt signaling. Notably, after mid-body amputation, these processes are unilateral, since they involve only the basal fragments and not the distal ones (Jeffery and Gorički 2021).

Brain regeneration was first described in 1964 (Lender and Bouchard-Madrelle 1964). Recently, a combination of several methods, including live imaging and functional analyses, along with transgenic animals expressing GFP in most neurons (Dahlberg et al. 2009), revealed that proliferating cells (a potential blastema) accumulated around severed nerve endings. The source of these cells, however, was not identified. The authors speculated that these cells could be progenitor cells already present in the central nervous system since the movement of GFP-positive cells along the axons or migration of undifferentiated cells from other body parts were not detected by confocal time-lapse microscopy. A recent study, however, reports the involvement of proliferating cells, originating in the branchial basket, in brain regeneration (Jeffery 2019). These candidate stem cells supply progenitor cells for regeneration and differentiate into hemocytes, neural, and muscle cells (Jeffery 2019).

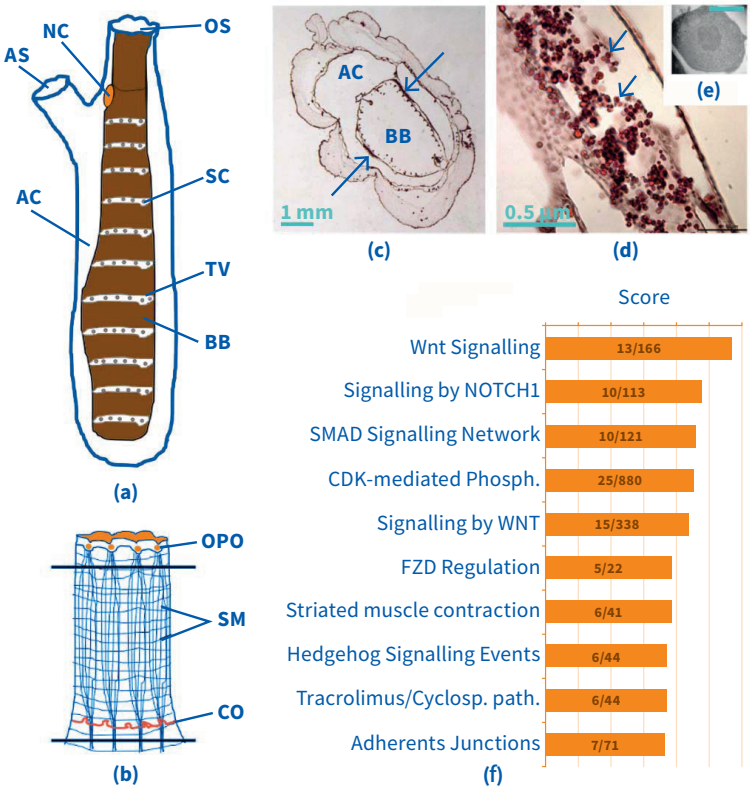

Figure 4. Regeneration and enriched pathways for highly expressed genes associated with stem cell activity in the solitary ascidian *Ciona robusta* endostyle: (**a**) illustration of a young adult individual (sagittal view, dorsal side at left). The branchial basket is perforated by numerous stigmata, delimited by longitudinal and transverse bars where hemocytes flow in vessels; (**b**) illustration of oral siphon. Upper horizontal line: siphon tip; lower horizontal line: siphon base; (**c**) transverse histological section of a juvenile individual (hematoxylin–eosin). Note that in transverse vessels there are hemocyte aggregations (arrows); (**d**) histological section of the same individual shown in (**c**) (hematoxylin–eosin). Detail of hemocytes (arrowheads) in a branchial basket transverse vessel; (**e**) hemoblast, transmission electron microscopy of a juvenile. The hemoblast was recognized at branchial basket level. Scale bar: 2 mm; (**f**) enrichment scores of the top ten pathways of annotated genes in endostyle using GeneAnalytics tool. The gene list used in the analysis is based on all the genes expressed by in situ hybridization (Ogasawara et al. 2002); overall, 185 genes expressed in endostyle were analyzed. In the bars, the number refers to annotated genes out of the list from the total genes in the human indicated pathways. AC: atrial chamber; AS: atrial siphon; CO: coronal organ; G: gut; NC: neural complex; OPO: oral siphon pigmented organ; OS: oral siphon; TV: transverse vessel; SM: siphon muscles; SC: stem cell. Source: Graphic by authors.

5.2. Development, Regeneration, and Stem Cells in Colonial Ascidians

Colonial ascidians possess extreme regenerative capacity known as vascular budding (Sabbadin et al. 1975) or whole-body regeneration (WBR) (Pancer et al. 1995), in which entire colonies regenerate from an aggregation of candidate stem cells in the vasculature (Pancer et al. 1995; Rinkevich et al. 2007a, 2007b; Voskoboynik et al. 2007; Manni et al. 2019; Kassmer et al. 2020). As described above, stem cells have been proven to mediate asexual reproduction in *B. schlosseri* (Laird et al. 2005) and, therefore, most likely also mediate vascular budding in this species.

5.2.1. Embryonic Development

In colonial ascidians, embryos develop inside adult zooids (or outside the parent body, isolated in the tunic) and, depending on the species and the temperature of the water, are released into the water as mature swimming larvae after about a week (Manni et al. 1993; Burighel and Cloney 1997; Winkley et al. 2019). During this development, embryonic stem and progenitor cells divide and generate the primary germ layers (ectoderm, mesoderm, and endoderm). At the morula and blastula stages, a tissue-specific molecular signature can already be detected (e.g., germline, endostyle, nervous system), and these systems subsequently form a swimming larva (Kowarsky et al. 2021). The hatched larva settles and metamorphoses into a sessile oozooid. During *B. schlosseri* embryogenesis, a bud develops within the larva and remains after metamorphosis in the oozooid, initiating asexual reproduction (astogeny) to produce a colony of genetically identical zooids.

The contemporary presence of disparate reproductive strategies (i.e., embryogenesis and blastogenesis) that generate similar individuals (an oozoid from a zygote and zooids from stem cells and progenitor cells), allows colonial ascidians to serve as valuable models to study how stem cells mediate developmental processes (Laird et al. 2005; Manni et al. 2006; Rosner et al. 2014; Voskoboynik and Weissman 2015; Kowarsky et al. 2021). In this context, the origin of hematopoietic stem cells (HSCs) and germline stem cells (GSCs) during embryogenesis and blastogenesis of *B. schlosseri* are of significant research interest (Rinkevich et al. 2013; Rosental et al. 2018; Kowarsky et al. 2021).

HSCs are multipotent stem cells that produce all blood cells in mice and humans (Spangrude et al. 1988). In *B. schlosseri*, candidate HSCs and progenitor cells have been identified, while the endostyle has been identified as their niche (Voskoboynik et al. 2008; Rinkevich et al. 2013; Rosental et al. 2018). During embryogenesis, hemoblasts (undifferentiated cells with a high nucleus–cytoplasm ratio, abundant ribosomes, and cytoplasm with few organelles), and morula cells appear in the early tailbud stage (Kowarsky et al. 2021). By the mid-late tailbud stage, hyaline amoebocytes and pigment cells appear. Macrophage-like cells appear at metamorphosis, and nephrocytes are found in the oozooid. The number of *B. schlosseri* HSC-associated

genes present during embryogenesis increases early in development (from the two-cell stage to the morula stage). This gene expression profile includes 239 homologous genes that are known to be expressed in the human hematopoietic bone marrow and 43 with human homologs expressed in HSCs (Kowarsky et al. 2021).

GSCs are the source of the gametes that produce daughter stem and differentiated cells through asymmetric cell division (Spradling et al. 2011). In vertebrates, GSCs segregate early in development producing a small founding population (Ueno et al. 2009). During *B. schlosseri* embryogenesis, cGSCs expressing *vasa* were identified in the early cleavage stage (Brown et al. 2009). Upon isolating the *B. schlosseri* cell populations by FACS, one cell population significantly upregulated 235 genes that were known to be enriched in mammalian germline (Rosental et al. 2018). The genes expressed by this cell population were used in the developmental atlas created by Kowarsky et al. (Kowarsky et al. 2021), to track germline development. The enrichment in GSC-associated genes suggests that, in the embryo, cGSCs develop during the morula stage and proliferate as embryogenesis proceeds. The same study compared the molecular signatures of cHSCs and cGSCs during the embryogenesis and blastogenesis pathways, revealing that both developmental pathways share similar patterns of HSC- and GSC-associated gene enrichment. The same was confirmed for tissue-specific signatures during embryogenesis and blastogenesis. This common trend suggests that tissue-specific stem cells mediate organogenesis with similar molecular dynamics during both sexual and asexual reproduction (Rosner et al. 2019; Kowarsky et al. 2021).

5.2.2. Asexual Reproduction

Colonial ascidian species produce their adult body through asexual reproduction by budding, in a process termed blastogenesis. During embryogenesis, embryonic stem cells differentiate and divide to build the complex adult body of the colony founder, the oozooid. During blastogenesis, asexual reproduction utilizes adult stem cells to clone new bodies and organs. As mentioned above, oozooids derived from metamorphosed larvae carry buds, the precursors for the next generation's zooid. Pharyngeal, stolonal, epicardial, palleal, and vascular budding are various types of blastogenesis described in colonial ascidians (Table 3; Figure 5).

In palleal budding, buds grow out from the body wall, specifically from the epidermis, the epithelium of the peribranchial chamber, and the connective tissue lying between them (Manni et al. 2007, 2014). Morphological studies show that buds form a double vesicle where the outer leaflet will differentiate into the epidermis, while the inner leaflet, originally derived from the peribranchial epithelium, will develop most of the zooidal tissues. This budding mode is used by colonial stolidobranch ascidians and has been mainly studied in *B. schlosseri*.

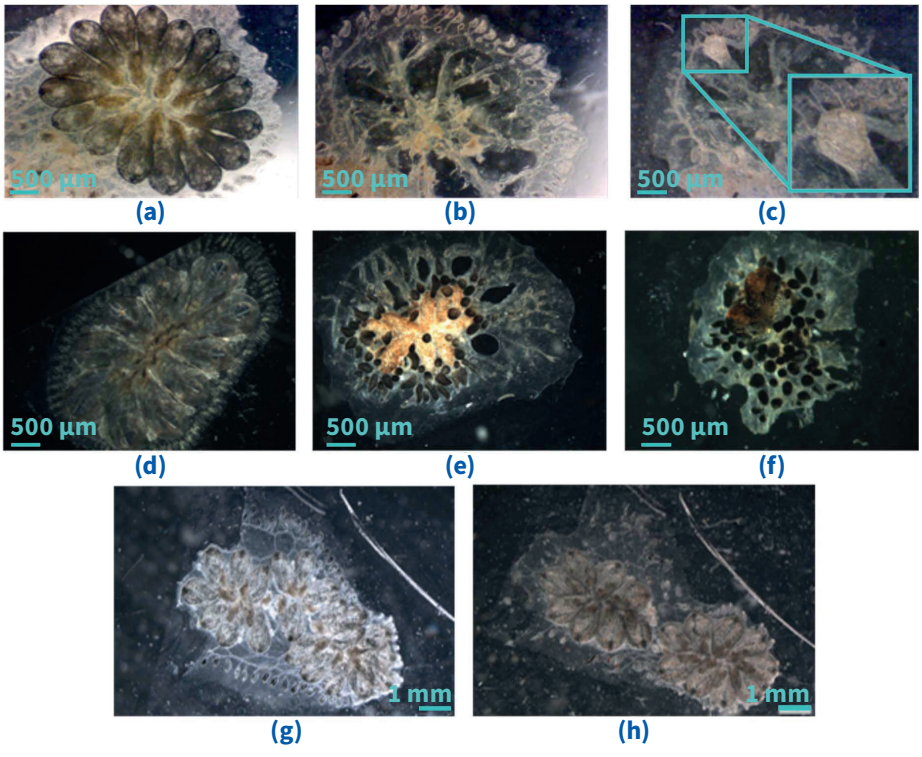

Figure 5. Regeneration in *B. schlosseri*. (**a**–**c**) WBR: (**a**) colony before the surgical manipulation; (**b**) colony after the removal of all the zooids of the colony, only the marginal vessel and ampullae are left; (**c**) colony after 5 days from the operation, with an enlargement of the developing vascular bud. (**d**–**f**) Budectomy induced WBR: (**d**) control colony in which no buds were removed; (**e**) colony after 6 days following the removal of all the buds. When takeover starts, the zooids are only partially resorbed through an attenuated apoptotic process. Tight aggregates of partially absorbed zooids and ampullae are formed. Then, new sporadic transparent elements appear in various sites in the colonial tunic, the new centers of regeneration; (**f**) 20 days after budectomy, functional zooids are differentiated from these regenerating sites. (**g**,**h**) circulatory system regeneration: (**g**) colony after the removal of a part of the marginal vessel and associated ampullae; (**e**) colony 3 days following partial blood vessel removal. Source: Graphic by authors.

Table 3. Prospective involvement of candidate stem cells in colonial ascidians asexual reproduction modes.

Asexual Reproduction Mode	Species	Candidate Stem Cell Identified and (Methods Used to Identify It)	References
Peribranchial budding	*Botryllus schlosseri*	Multipotent peribranchial epithelia and candidate circulating stem cells (transplantations, labeling, long-term lineage tracing).	(Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013; Rosental et al. 2018)
	Botrylloides violaceus	Candidate circulating vasa expressing cells in buds and vasculature system (ISH)	(Brown and Swalla 2007)
Vascular budding	*Botrylloides leachii/diegensis*	Piwi-positive candidate stem cells lining the vascular epithelium (ISH)	(Rinkevich et al. 2010)
Epicardial budding	*Diplosoma listerianum*	Cells proliferating in the adult and in the bud and high telomerase activity in the buds (BrDU; TRAP)	(Sköld et al. 2011)
Stolonal budding	*Perophora viridis*	Mesenchymal cells form gonads, heart, and cerebral ganglion (morphological studies)	(Lefèvre 1897, 1898)
	Clavelina lepadiformis	Mesenchymal cells form gonads and the nervous system (morphological studies)	(Brien and Brien-Gavage 1928; Brien 1968)

Vascular budding is another budding mode present in colonial stolidobranch ascidians, which occurs under normal conditions or in the aestivation of botryllid colonies. It was first recorded by Savigny (1816) and Giard (1872). Morphological studies suggest that new zooids regenerate from aggregated cells (hemocytes contacting the epidermis lining the hemolymphatic vessels) with the morphological features of undifferentiated cells, such as a small diameter and large round nuclei with packed chromatin (Oka and Watanabe 1957a; Freeman 1964).

Stolonal budding characterizes Clavelinidae and Perophoridae growth (Figure 1k,l). In these taxa, buds develop from the stolon, an outgrowth of the zooid body that connects individual zooids keeping them attached to the substrate. The stolon is bordered by the epidermis and contains 2–3 sinuses (known as vessels), separated by connective tissue (Kott 2001). In *Perophora*, mesenchymal cells accumulate in the growing extremity of the stolon, where they proliferate and

develop the bud inner vesicle (Brien and Brien-Gavage 1928; Koguchi et al. 1993). Morphological studies suggest that the outer vesicle originates from the epidermis and will continue to form epidermal layers, while the inner vesicle develops the peribranchial and branchial chambers, as well as the neural gland, gut, and endostyle. Circulating hemocytes participate in the formation of *Perophora's* gonads, heart, and brain (Lefèvre 1897, 1898). Even in Clavelinidae's stolonial budding, mesenchymal cells (also called neurogenital mass) are suggested to be involved in the development of the nervous system and the germline (Brien and Brien-Gavage 1928; Brien 1968). A particular type of stolonal budding, called vasal budding, was recently described in the stolidobranch ascidian *Polyandrocarpa zorritensis* (Figure 1j) (Scelzo et al. 2019). Buds originate from the thickening and invagination of a patch of cells on the epidermis. The invagination leads to the formation of a double vesicle (outer and inner epidermis and the hemolymph between them). Since aggregations of hemoblasts are observed around the forming inner vesicle, it has been suggested that circulating cells also contribute to organogenesis in this budding mode (Scelzo et al. 2019; Alié et al. 2021).

Epicardial budding or strobilation characterizes most colonial Aplousobranchia. In this process, buds derive from epidermal constrictions that enclose part of the epicardium, a tube-like sac originating as an invagination of the pharynx (Sunanaga et al. 2008) and other tissues. Sköld et al. (2011) observed an extensive cell proliferation in growing epicardial buds of *Diplosoma listerianum*.

5.2.3. Whole-Body Regeneration

While some colonial ascidians species continuously develop zooids from their vasculature (Oka and Watanabe 1957a; Freeman 1964; Saito and Watanabe 1985; Okuyama and Saito 2001; Gutierrez and Brown 2017) (Figure 1k,l), other colonial species regenerate the whole body from their vasculature only when injured (e.g., after zooid- and budectomy). This kind of regeneration is known as whole-body regeneration (WBR). *B. schlosseri*, *Botrylloides leachii*, *Botrylloides violaceus*, and *Botrylloides diegensis* are among the species used for WBR studies (Table 4).

In *Botrylloides* species, WBR occurs in isolated fragments of colonial matrix and vasculature (Rinkevich et al. 1995, 2007a, 2007b, 2008; Brown and Swalla 2007; Kassmer et al. 2020). In *B. schlosseri*, WBR can be induced by removing all the individuals from colonies approaching the cyclical generation change or takeover (TO) (Voskoboynik et al. 2007), during which massive apoptosis events occur in adult tissues (Lauzon et al. 1992, 2007; Cima et al. 2010) that are resorbed and succeeded by their primary buds (Figure 5a–c**)**. In this case, WBR requires an intact marginal hemolymphatic vessel of the colony (Milkman 1967; Sabbadin et al. 1975; Voskoboynik et al. 2007; Kürn et al. 2011; Ricci et al. 2016).

Table 4. Regeneration capacity and involvement of candidate stem cells in colonial ascidian regeneration. N/A: not available.

Regenerative Structure	Candidate Stem Cell Description and Identification	Candidate Stem Cell Source	References
Botryllus schlosseri			
Vessels and ampullae	Preexisting vascular tissue-resident cells. Based on vascular cell lineage tracing	Vascular tissue	(Zaniolo and Trentin 1987; Gasparini et al. 2008, 2014; Tiozzo et al. 2008b; Braden et al. 2014)
Whole-body from colonial vasculature	N/A	Hemolymph/colonial vasculature	(Sabbadin et al. 1975; Voskoboynik et al. 2007; Ricci et al. 2016)
Whole-body from body fragments	N/A Circulating cells expressing Pl10	Tissue fragments Hemolymph	(Sabbadin et al. 1975; Majone 1977; Rosner et al. 2019)
Vessels and ampullae	Preexisting vascular tissue-resident cells. Based on vascular cell lineage tracing	Vascular tissue	(Zaniolo and Trentin 1987; Gasparini et al. 2008, 2014; Tiozzo et al. 2008b; Braden et al. 2014)
Botrylloides leachii, *Botrylloides diegensis*			
Whole-body from colonial vasculature	Candidate stem cells expressing *Piwi*. Based on inhibition of WBR upon injection of siRNA for *Piwi*	Vasculature epithelia	(Rinkevich et al. 1995, 2007b, 2008, 2010; Zondag et al. 2016, 2019)
Botrylloides violaceus			
The whole body from the colonial vasculature	Candidate stem cells in the vasculature expressing Integrin alpha 6. Based on regeneration recovery on colonies treated with mytomycin C and injected with one IA6+ cell and on lineage tracing (EdU)	Circulatory hemocytes	(Brown et al. 2009; Kassmer et al. 2020)
Polyandrocarpa zorritensis			
Whole body	N/A	Hemoblasts, based on morphological data	(Scelzo et al. 2019)

An alternative mode of WBR described in *B. schlosseri* was termed budectomy-induced WBR (Figure 4d–f) (Rosner et al. 2019). Notably, 100% of young colonies (<6 months old) and 50–60% of old colonies (>8 months old) form new zooids within 2–3 weeks following complete budectomy. In this case, adult zooids regularly enter

the programmed TO phase. However, the apoptosis process does not culminate in the complete removal of the zooids' debris. Instead, some cells in the degenerating zooids or in the vascular vessels start proliferating to form new zooids. The presence of even a single bud in the colony prevents this mode of regeneration and leads to the full resorption of the zooidal generation and the survival of the single bud, which can reform the colony.

Several members of the *IAP* family of genes, the *PI3K/Akt* pathway, apoptosis signals, as well as signals derived from the buds themselves, are involved in the regulation of this mode of regeneration (Rosner et al. 2019). The tight association between the onset of apoptosis and regeneration may be attributed to a phenomenon called apoptosis-induced compensatory proliferation that has been described in additional animal models (invertebrates and vertebrates) (Bergmann and Steller 2010; Fan and Bergmann 2008). During this process, *caspase 3* activates target genes in a *Xiap* (or its ortholog)-dependent manner.

In stolonal species, such as *P. zorritensis* (Figure 1j), *Clavelina lepadiformis*, and *Perophora viridis* (Figure 1k,l), WBR is induced when part of the stolon is isolated from the remaining colony (Della Valle 1914; Huxley 1921; Brien 1930; Deviney 1934; Ries 1937; Goldin 1948; Scelzo et al. 2019). WBR in *Symplegma reptans*, *P. misakiensis*, and *B. schlosseri* can also occur from isolated bud fragments able to produce new buds before being slowly resorbed (Majone 1977; Sugino and Nakauchi 1987).

In *B. violaceus* (Brown et al. 2009) and *B. leachii* (Rinkevich et al. 2010) WBR, hemocytes adhering to the vasculature epithelium express *piwi*. In *B. violaceus*, the piwi-positive cells show immunopositivity to anti-PCNA antibodies (Brown et al. 2009). Retinoid acid (RA) is required for *B. leachii* WBR: the presence of RA agonist increases the number of buds, whereas RA inhibitors block the process (Rinkevich et al. 2007a). In addition, the development of regenerating buds in *B. leachii* is altered by serine protease inhibitors, suggesting a role to this enzyme during regeneration (Rinkevich et al. 2007b).

Candidate stem cells expressing *integrin-alpha-6* (IA6), *pou3*, and *vasa* have been suggested to mediate WBR in *B. diegensis* (Kassmer et al. 2020). In fragmented *B. diegensis* tissues that were treated by mitomycin C, WBR was triggered by transplantation of a single $IA6^+$ cell. Moreover, when both Notch or canonical Wnt signaling pathways were impaired by treatment with specific drugs, WBR could not be triggered through transplantation of $IA6^+$ cells, suggesting that these pathways play an important role in the process (Kassmer et al. 2020).

This study suggests that a single $IA6^+$ candidate stem cell can mediate WBR in *B. diegensis*. However, long-term tracing of transplanted cells to fully understand their differentiation potential, along with reisolation and transplantation of $IA6^+$ cells from primary recipients to secondary hosts with the same WBR outcomes, will be needed

to clarify whether these cells are stem cells, and what is their potency potential (e.g., multipotent, pluripotent cells).

5.2.4. Tunic and Colonial Circulatory System Regeneration

Colonial circulatory system regeneration (CCR) refers to the ability of a colony to regenerate its tunic and the circulatory system following damage. Ascidians possess an open circulatory system containing diverse cells, flowing in hemolymphatic spaces and in sinuses and lacunae of the body wall, delimited by connective tissues (Millar 1953; Kriebel 1968; Monniot et al. 1991). Some colonial ascidians have a system of vessels that cross the tunic and connect between zooids. Tunic vessels originate from the zooid epidermis; therefore, they are not homologous to the mesodermal vertebrate blood vessels. CCR was studied in *B. schlosseri* (Figure 1g; Figure 5g,h), in which full regeneration occurs in a period of time ranging from a few hours to days, depending on the extent of the ablation or the stress that causes vessel degeneration (Zaniolo and Trentin 1987; Gasparini et al. 2008; Qarri et al. 2020; Tiozzo et al. 2008b; Braden et al. 2014). Damage caused by UV exposure to the vasculature was repaired within a few days (Qarri et al. 2020). The incubation with anti-PCNA antibodies revealed that the proliferation of epidermal cells occurs immediately after ablation, as these cells contribute to the synthesis of a new tunic (Gasparini et al. 2008). The regeneration of vasculature is stimulated by the injection into the circulatory system of vertebrate vascular endothelial growth factor (VEGF) and epidermal growth factor (EGF) (Gasparini et al. 2014). Both the knockdown of the VEGF receptor and the inhibition of VEGFR by a chemical agent inhibit vascular regeneration, suggesting the VEGF pathway plays a role in this process (Tiozzo et al. 2008b). Braden et al. (Braden et al. 2014) injected fluorophores to label the cells inside the vasculature in *B. schlosseri* and followed their contribution to vascular regeneration: they identified resident, proliferating cells that expressed homologs of *cd133*, *vegfr*, and *cadherin* and suggested that they contribute to vasculature regeneration.

5.2.5. In and Out of Dormancy

The ability of organisms to become dormant (termed torpor) when rough environmental conditions appear is well documented in marine invertebrates. Some taxa display seasonal torpor where hibernation occurs in the winter, and aestivation occurs in the summer (Storey and Storey 2011). Hibernation and aestivation events are recorded in a wide range of ascidians, including *C. lepadiformis* (De Caralt et al. 2002), several *Perophora* species (Mukai et al. 1983), *Polysyncraton lacazei* (Turon 1992), *Diazona* and *Aplidium* (Nakauchi 1982), *Ecteinascidia turbinata* (Carballo 2000), *Didemnum vexillum* (Valentine 2009), *Pseudodistoma crucisgaster* (Tarjuelo et al. 2004) and botryllid ascidians (Bancroft 1903; Burighel et al. 1976; Rinkevich and Rabinowitz 1993; Rinkevich et al. 1996; Hyams et al. 2017). The torpor states (hibernation/aestivation)

were studied in *Botrylloides leachii* on the Levantine coast of Israel (Rinkevich and Rabinowitz 1993; Hyams et al. 2017) and on the Italian coast of the Adriatic Sea (Bancroft 1903; Burighel et al. 1976). The role of stem cells in torpor states of *B. leachii* was first suggested (Rinkevich et al. 1996) as part of the survival budding repertoire of this species, which includes the WBR phenomenon (Pancer et al. 1995). Alongside hibernation, Hyams et al. (2017) revealed high expression levels of genes related to stem cell activity including *piwi*, *pl10,* and *pcna*, mostly by multinucleated cells, whose numbers were observed to increase during torpor in *B. leachii*. Using in situ hybridization and immunohistochemistry assays, Hyams et al. (2017) documented that *piwi Pl10* and *pcna* expressions during the hibernation processes diverged significantly from normal blastogenesis (asexual growth) related expressions. As the hibernation progressed, the cells that expressed *piwi*, *Pl10*, and *pcna* significantly increased in numbers, peaking in aroused colonies. In non-hibernating colonies, these markers are highly expressed in the cell islands stem cell niches along the endostyle (Rinkevich et al. 2013).

5.2.6. Stem Cell Aging

As described above, colonial ascidians undergo cyclical formation of new individuals (zooids) by stem-cell-mediated budding (Laird et al. 2005; Voskoboynik et al. 2008). In this cyclical process, zooids die through massive apoptosis as the next generation of buds matures into an entire new replicated zooid body. As the colony ages, both sexual and asexual reproduction methods slow and eventually halt, demonstrating the colonies' reduced regenerative potential (Voskoboynik and Weissman 2015). While the colony can live for years, the zooids live for only a few days, creating unique characteristics that distinguish it from aging in solitary organisms (Rosen 1986; Voskoboynik and Weissman 2015; Rinkevich 2017). The colony ages due to its stem cells that remain and circulate from one generation to the next. As new zooids are formed, the self-renewing stem cells are the cells that are maintained and age throughout the life of the colony (Voskoboynik and Weissman 2015).

Zooid death is part of the botryllids' life cycle and is not indicative of the systemic aging processes that occur within the colony (Borges 2009). A study on the weekly cycle of *B. schlosseri* zooids weekly cycle (Ben-Hamo et al. 2018) has revealed the importance of mortalin (an HSP70 family member that is highly associated with development, cell proliferation, senescence, aging, and apoptosis) for the zooid life cycle. In the planarian *Dugesia japonica*, *djmot*, the mortalin-like gene is expressed in the neoblasts—the adult stem cells of the animal (Conte et al. 2009).

In *B. schlosseri*, mortalin is highly expressed in the endostyle and putative circulating stem cells, and its expression is reduced in zooids during the takeover stage (Ben-Hamo et al. 2018). It is also expressed in in vitro epithelial monolayers

that also express other genes associated with stem cells (Rabinowitz and Rinkevich 2004, 2011).

Lifespans differ between wild *B. schlosseri* colonies grown in the field, compared with colonies reared in the lab. Colonies grown in the field have short, subannual life spans (Grosberg 1988; Chadwick-Furman and Weissman 1995a, 1995b) influenced by seasonal fluctuations of light, nutrients, and temperature. Spring-born colonies have a shorter lifespan of about 3 months, compared with the 8-month lifespan of fall-born colonies (Chadwick-Furman and Weissman 1995a, 1995b). Laboratory-bred colonies exhibit either short (<0.5 years), medium (0.5–2 years), or long (2–20+ years) lifespan (Sabbadin 1969; Boyd et al. 1986; Rinkevich et al. 1992; Lauzon et al. 2000; Voskoboynik and Weissman 2015; Rinkevich 2017; Voskoboynik et al. 2020). However, when an individual *Botryllus* colony is divided into several subclones (clonal replicates), the subclones will often die simultaneously (Rinkevich et al. 1992; Lauzon et al. 2000). This suggests that lifespan in *B. schlosseri* colonies is determined through a heritable factor. Morphological differences are observed in older colonies, such as increased pigmentation, reduced zooid size, and reshaping of the vasculature system (Voskoboynik and Weissman 2015; Voskoboynik et al. 2020; Rodriguez et al. 2021). The diurnal circadian cycle also differs in aged versus young and mid-aged colonies, with younger colonies exhibiting reduced nocturnal heart rate and siphon activity, while aged *B. schlosseri* colonies show no observable circadian changes/changes in heart rate and siphon activity, indicating that diurnal phenotypes diminish with age (Voskoboynik et al. 2020). Using a comprehensive transcriptome sequencing of whole systems, *B schlosseri* colonies were sampled every 3 h over a 24 h period. Samples from three different age groups (36–140 days; 2142–2146 days; 5869–5871 days) (Voskoboynik et al. 2020) revealed that the oscillation patterns of *B. schlosseri* clock and clock-controlled genes declined with age. Age-specific cyclical expressions were found in hundreds of pathways including those associated with known hallmarks of aging (Voskoboynik et al. 2020; López-Otín et al. 2013). Significant age-associated changes were found in the cycling dynamics of genes associated with the *B. schlosseri* enriched HSC and GSC, as well as the endostyle and the central nervous system (Voskoboynik et al. 2020).

A subsequent CNS study (Anselmi et al. 2021) characterized brains from diverse developmental stages and ages, discovering that each week the number of neurons in the zooid brain fluctuates, reaching a maximum of ~1000 cells, and thereafter decreasing while the number of immunocytes increases. Comparing the number of neurons in the brains of young and old colonies, they found that aged brains contain fewer cells. In both weekly degeneration cycles and overall *B. schlosseri* aging, they observed that the decrease in the number of neurons correlates with reduced response to stimuli and with significant changes in the expression of genes for which the mammalian homologous are associated with neural stem cells and neurodegeneration

pathways (Anselmi et al. 2021). Among the 411 putative homologous genes that correlate with neurodegenerative diseases (including Alzheimer's, Parkinson's, and dementia), that are expressed in the *B. schlosseri* brain, 71 are differentially expressed between early and late cycle, and 157 are differentially expressed between young and old colonies.

Since stem cells are the only cells that self-renew and are maintained throughout the entire life of the colony, the aging phenotypes described above most likely reflect tissue-specific stem cells exhaustion.

5.2.7. Stem Cell Competition in Development and Aging

As described above, colonial ascidians such as *B. schlosseri* may form natural chimeras with adjacent colonies by vascular fusion if they share one or two alleles in the highly polymorphic histocompatibility gene, BHF (Voskoboynik et al. 2013b). Itinerant GSCs compete in chimeras with heritable winner and loser hierarchies (Stoner et al. 1999; Laird et al. 2005; Rinkevich et al. 2013). These studies revealed fundamental aspects of stem cell biology with relevance to pathological conditions in humans (Weissman 2000, 2015). Studying mammalian stem cells as clones of competing stem cells, the Weissman lab and others discovered that competition between stem cells led to the emergence of myeloid biased HSC clones that dominate aged mice and humans and produced mainly cells from the myeloid lineage when compared to young animals where balanced HSC clones produce cells from both lymphoid and myeloid lineages (Rossi et al. 2005, 2007; Beerman et al. 2010; Pang et al. 2011, 2013). Stem cell competition is also observed in human acute myeloid leukemia where clonal preleukemic progression occurs in the HSC stage and each heritable change increases the competitive competence of the clone vs. normal HSC (Jamieson et al. 2004, 2006; Miyamoto et al. 2000; Jan et al. 2012; Corces-Zimmerman et al. 2014; Jaiswal and Ebert 2014; Sykes et al. 2015), and amongst germline stem cells (Ueno et al. 2009). Understanding the molecular determinants that regulate stem cell competition and the expansion of specific clones throughout an organism's life is now a major area of interest in stem cell aging and cancer and regenerative medicine (Weissman 2015).

6. Adult Stem Cell Niches

The term stem cell niche, originally conceptualized by (Schofield 1978), refers to a discrete anatomical microenvironment where stem cells and their milieu reside, all playing critical roles in maintaining/regulating the stem cell state and self-renewal potential (Fuchs et al. 2004; Saez et al. 2017). Morphologically, all niches hold self-renewal stem cells and their progeny, heterologous cell types, and the surrounding niche-specific extracellular matrix (Chacón-Martínez et al. 2018). Consistent with the strict vertebrate definition in which stem cells are present in their undifferentiated

and, in some cases, quiescent states, the vast majority of stem cell niches in ascidians are putative.

6.1. Somatic Stem Cell Niches

In ascidians, prospective stem cell niches have been identified in solitary and colonial species. As previously reported in *Ciona*, putative stem cells residing in pharyngeal sinuses and lymph nodules migrate to the distal regeneration blastema (Figure 4). A short pulse of the DNA synthesis marker 5-ethynyl-2'-deoxyuridine (EdU) labels dividing cells located in the pharyngeal sinuses, while EdU pulse–chase results in the regenerating oral siphon, in an area resembling a blastema. These cells were labeled by anti-piwi antibodies and expressed alkaline phosphatase activity, which is associated with stem cells (Auger et al. 2010; Jeffery 2015b). Furthermore, EdU-labeled cells were observed in *Ciona*-regenerating oral siphons following transplantation of branchial sac fragments, taken from EdU-treated *Ciona* to recipients that were not treated by EdU, but their oral siphon was removed. These results demonstrate the presence of proliferating cells that migrate to regenerating sites and are involved in tissue regeneration (Jeffery 2015b, 2019). Additional putative stem cell niches harboring hemoblast-like cells have been identified in the pharyngeal nodules of *Styela clava* and in the intestinal submucosa of *Styela plicata* (Ermak 1976; Jiménez-Merino et al. 2019).

Using in vivo cell labeling, transplantation experiments, confocal microscopy, and time-lapse imaging, Voskoboynik et al. (2008) found cells with stem cell potentiality in the anterior ventral region of the *B. schlosseri*'s endostyle (subendostylar sinus) (Figure 6a–d). Cells from the endostyle niche divide and migrate to developing organs in buds but do not participate in gonads formation. When a few cells are transplanted from the endostyle niche, they participate in tissue formation and induce long-term chimerism in allogeneic tissues. When a few cells are transplanted from the vasculature, they do not contribute to tissue formation or induce chimerism. Being able to label and monitor cells in vivo by imaging them in their natural niches through the transparent body of this model organism, in combination with the ability to transplant cells between allogeneic colonies, provides a fundamental framework to trace cell differentiation into more mature cell types for studying stem cell development (Voskoboynik et al. 2008). This study was the first to demonstrate the endostyle's role beyond assisting in feeding (secretes mucus) and iodine accumulation (homologous to vertebrate thyroid). Supporting the subendostylar niche as a somatic stem cell niche, a decade later it was shown, through cHSC transplantations and diverse functional essays, that the *B. schlosseri*'s subendostylar niche is harboring cHSC. Furthermore, the endostyle molecular signature further suggested that the vertebrate hematopoietic bone marrow niche evolved from an organ resembling the *B. schlosseri* endostyle (Figure 6e) (Rosental et al. 2018). Specifically, this analysis

revealed 337 shared genes with significant upregulation between the *B. schlosseri* endostyle and human hematopoietic bone marrow. These include the genes *foxo3*, needed for hematopoietic stem and progenitor cell maintenance; *notch1*; *smad2*, important for adult murine HSC function; *vwf*, the von Willebrand factor. Analyses of genes expressed in *C. robusta* endostyle based on in situ expression data (Ogasawara et al. 2002) revealed significant similarities with the genes expressed in the endostyle of *B. schlosseri* (Figure 4g) (Rosental et al. 2018). Importantly, other stem cell niches could exist, including ampullae that can regenerate a whole zooid (Voskoboynik et al. 2007) and niches in the branchial sac sinuses (Jeffery 2015a). Considering that many of the candidate stem cell niches found in solitary and colonial ascidian species are associated with sinuses and cells aggregations located in proximity to the branchial sac (e.g., endostyle niche/cell islands/nodule/lymph nodes) a comprehensive study aiming to compare these candidate stem cell niches may reveal conserved elements essential for stem cell maintenance.

6.2. Germ Stem Cells and Their Niches

The oocytes of solitary and colonial ascidians contain a special region called postplasm (Shirae-Kurabayashi et al. 2006; Brown et al. 2009; Rosner et al. 2009), which holds the condensed aggregate of maternal RNA and protein molecules, similar in content and functionality to the germ plasm observed in some organisms (e.g., *C. elegans*, *Drosophila*, Zebrafish). In these organisms, with preformistic modes of germline sequestering, the cells committed to becoming primordial germ cells (PGCs) inherit the germ plasm-like derived maternal components, limiting cell differentiation into the germ lineage to the cell's descendants. In *C. robusta*, postplasm was identified in the posterior-most blastomeres and thereafter in B8.12 cells that were classified as PGCs formed in a postplasm-dependent manner (Wessel et al. 2020). In larvae, those PGCs reside in the ventral side of the tail until metamorphosis, when the PGCs are retracted along with tail tissues into the body trunk and populate the gonads (Shirae-Kurabayashi et al. 2006).

In colonial ascidians, a cGSC-specific transcriptomic signature suggests that PGCs are established at the embryo's morula stage (E1.4) and proliferate as the embryo grows (Kowarsky et al. 2021). At this stage, candidate germ precursor cells expressing vasa were identified (Brown et al. 2009), with candidate PGCs identified in the embryo body trunk but not observed within the tail (Rosner et al. 2009). Examples of postplasm materials that are important for PGC specification include *pem*, *piwi,* and *vasa* gene products. In *C. robusta*, *pem* functions to repress somatic specific gene expressions in early germline at the level of polymerase II (Pol II) activity (Strome and Updike 2015). Pem proteins are only transiently expressed during the early specification of germ cells, while during later stages, their function is replaced by a chromatin repression mechanism (e.g., in *Halocynthia roretzi*) (Zheng et al. 2020).

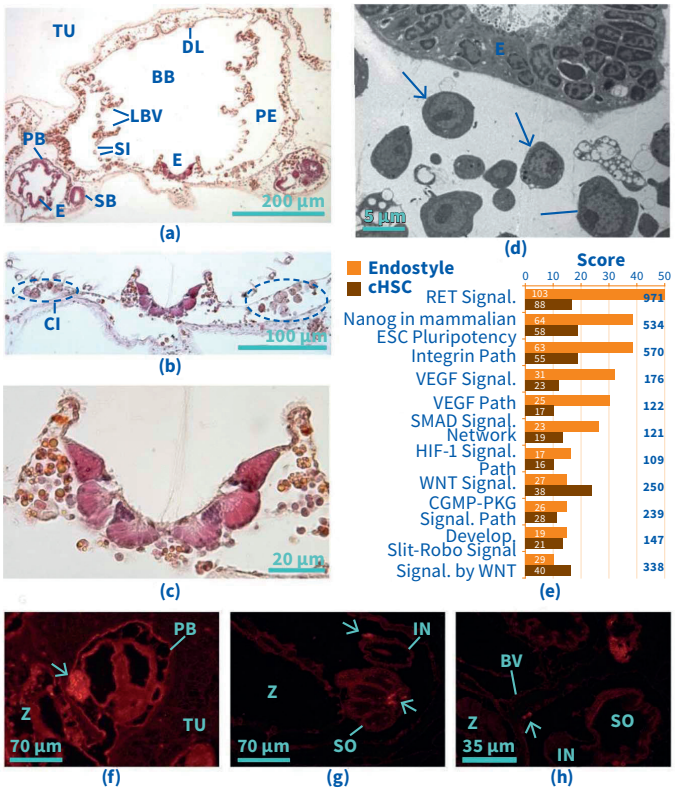

Figure 6. Putative stem cells and stem cell niches in *B. schlosseri*: (**a**–**c**) endostyle and cell island niches. Dotted lines in b: cell islands; (**d**) hemoblasts (arrows) in endostyle niche, transmission electron microscopy; (**e**) enrichment scores of pathways associated with stem cell activity that are expressed in *B. schlosseri* endostyle and enriched HSCs using GeneAnalytics tool. The gene list used in the analysis is based on gene expression data of isolated endostyles and enriched *B. schlosseri* HSCs populations described in Rosental et al. (2018). The number in the bars indicates the number of genes that were significantly upregulated in endostyle/cHSC populations and annotated to human genes in the specific pathway. The numbers on the right indicate the total number of genes in the specific pathway known in humans. Bars indicate the score of the pathway; high and medium scoring pathways associated with stem cells that appear in both gene sets (endostyle and HSCs) were used; (**f**–**h**) immunohistochemical analyses of fixed sections with cy3+coupled BS-Vasa polyclonal antibodies: (**f**) staining of the gonad (arrow) within a primary bud; (**g**) vasa-positive cells aggregate attached to the zooidal stomach and intestine (arrows); (**h**) vasa-positive cells aggregate in the hemolymphatic vessel (arrow) and attach to the zooidal stomach. BB: branchial basket; BV: blood vessel; CI: cell island; DL: dorsal lamina; E: endostyle; LBV: longitudinal branchial vessels; IN: intestine; PB: primary bud; PE: peribranchial chamber; SB: secondary bud; SI: stigmata; SO: stomach; TU: tunic; Z: zooid. Source: Graphic by authors.

Vasa is considered a key marker of PGCs and germ lineages, although its expression was also detected in the somatic cells of various aquatic animals, including ascidians (Rosner et al. 2009). Vasa is an RNA helicase involved in the remodeling of RNA structure and in the regulation of genes translation. Moreover, vasa protein acts on the piwi-interacting RNA (piRNA) metabolic process and, together with piRNA and piwi proteins, governs the transposons methylation needed for their repression to ensure germline integrity (Siomi and Kuramochi-Miyagawa 2009; Kuramochi-Miyagawa et al. 2010). As such, *vasa* and *piwi* expressions were studied in many solitary and colonial ascidians (Fujimura and Takamura 2000; Shirae-Kurabayashi et al. 2006; Brown et al. 2009; Rosner et al. 2009, 2013). In *B. primigenus*, when vasa-expressing cells were depleted from the colony, vasa-expressing germ cells reappeared in the colony to form piwi-expressing candidate germ stem cells (Kawamura and Sunanaga 2009).

Studies suggest that in some marine invertebrate taxa with high regenerative aptitude (e.g., sponges, hydrozoans, and planarians), the adult stem cells can differentiate into both germ and somatic lineages (Buss 1982, 1983; Blackstone and Jasker 2003; Extavour and Akam 2003; Juliano et al. 2010; Alié et al. 2015; Fierro-Constaín et al. 2017; Rosner et al. 2021).

Considering the high regenerative capacity of ascidians, the potential for ascidian adult stem cells to differentiate into germ lineage in an alternative parallel mode of PGC sequestering has been investigated in both solitary and colonial species. In *C. robusta*, PGCs removal by cutting larval tails is compensated by the regeneration of the germ cells from cells that otherwise are assumed to have a somatic fate (Takamura et al. 2002; Yoshida et al. 2017; Wessel et al. 2020). However, since the cells behind this phenomenon were not identified yet, it is not clear whether this mode of sequestering is restricted to a specific time window during development or if PGCs regenerate from soma/germ stem cells or by trans- or de-differentiation of other cells. Experiments performed with TALEN-induced mutations in germ lineage (Yoshida et al. 2017) suggest that this mode of induction might occur without the removal of the original PGCs, and the cells involved in this process might be of epidermal, neural, muscle, or stem cell origin. Opposing conclusions were drawn by Laird et al. (2005) working with *B. schlosseri* and tracing the fate of single cells transplanted into genetically distinct individuals. This research, which was further strengthened by Voskoboynik et al. (2008) and Rinkevich et al. (2013), implies that cells with self-renewing and differentiation abilities can differentiate into somatic or germ cells but not both. These opposing results might reflect differences between species or even solitary versus colonial variations. However, single-cell lineage tracing experiments are needed to solve this discrepancy.

No matter the mode of germ cell sequestering, the germ cell precursors are always formed earlier than the gonads and the PGCs and migrate (passively or

actively) to the gonad, which might be relatively far apart from the PGCs. PGC motility is associated with the regulation of the level of their adhesion molecules at the onset and end of the movement and acquisition of amoeboid movement during the migration (Grimaldi and Raz 2020). Molecules that were associated with this movement include G protein-coupled receptors and Dead-end protein (Dnd), an RNA-binding protein involved in cell survival and fate that regulate proteins of the "motility module" (Grimaldi and Raz 2020). Colonial ascidians are characterized by repeated weekly migration of PGCs to the gonads of the newly formed buds. There, apart from the gonads situated in the buds that serve as niches for the germ lineages, it seems that additional "temporary niches" exist in various zooidal tissues including the cell islands (Figure 6b) (Rinkevich et al. 2013; Rosner et al. 2013). In *B. schlosseri*, cell islands were identified as niches for putative germ stem cells (Rinkevich et al. 2013). Expression of genes associated with germ and general stem cells was shown within them. These include *piwi*, *alkaline-phosphatase*, *vasa*, *pl10*, and *pcna*. Transplantation of whole-cell islands induces chimerism in the gonadal tissues. Moreover, labeling of cells in the cell islands leads to the appearance of the stain 10 days later in the gonads, including testis and ovaries of the newly developed zooids (Rinkevich et al. 2013). Isolated by cell sorting, a candidate GSC population, uniquely expressed 80 genes known to be expressed in mammalian germline, migrated to the cell islands following transplantation (Rosental et al. 2018), providing more support to the identification of the cell islands as a germline stem cell niche. Migrating PGCs in *B. schlosseri* were defined as BS-Vasa^{+}-BS-DDX1+BS-cadherin^{+}-γ-H2AX^{+}-phospho-Smad1/5/8^{+} cell aggregates (Rosner et al. 2013). These PGCs form complexes, mediated by BS-cadherin (Rosner et al. 2013), with follicular cells expressing members of the TGF-b family, which are the migratory unit during PGCs migration to the gonads (Langenbacher and Tomaso 2016). Additionally, changes in the migration of germ cells between old and new gonads in the new generation of buds are due to a chemotactic signal along a sphingosine-1-phosphate gradient (Kassmer et al. 2015) and involves also an ABC transporter-mediated autocrine export of an eicosanoid signaling (Kassmer et al. 2020).

7. Stem Cells as a Unit of Natural Selection

Discussing chimerisms in slime molds, Buss (1982) hypothesized that cells can compete within a chimera and take it over. By studying chimeras of the colonial chordate *Botryllus schlosseri,* the Weissman group discovered that natural selection operates at the level of GSC clones, which compete for niches within the organism's body. Chimeras usually produce only GSC's from one chimeric partner, despite maintaining the soma of both, leading to reproductive pressures toward increasingly competitive GSC's (Stoner and Weissman 1996; Stoner et al. 1999; Weissman 2000, 2015; Laird et al. 2005; Rinkevich et al. 2013). Weissman (2000) further suggested that stem

cells are not only units of biological organization, responsible for the development and the regeneration of tissue and organ systems, but are also units in evolution by natural selection. On the other hand, considerations of somatic adult stem cells of animals, including cancer stem cells (another type of adult stem cell not discussed here; Greaves 2013) as units of selection are not trivial, because of the failure to identify the hierarchical level upon which natural selection operates and what exactly is being selected (Rinkevich 2000; Greaves 2013). Indeed, in vertebrates and ecdysozoan invertebrates, adult stem cells are observed as pools of undifferentiated cells capable of self-renewal, proliferation, and production of a number of differentiated but lineage-restricted progenies, all for the general maintenance and various regeneration needs. Yet, the literature on non-ecdysozoan invertebrates (e.g., Gremigni and Puccinelli 1977; Rinkevich et al. 2007b; Ereskovsky et al. 2015; Hyams et al. 2017; Ferrario et al. 2020) suggests that adult stem cells carry a great degree of plasticity in their functions; therefore, the tissue-specific and lineage-restricted adult stem cell view, mainly derived from studies on vertebrates, may need to be expanded. Flexibility in the adult stem cell destiny allows high capabilities for regeneration and changes in cell fates in response to any emerging need; however, experiments that enable long-term lineage tracing of a single cell must be employed before conclusions regarding cell plasticity are made.

In many animal taxa (including sponges, cnidarians, and platyhelminths), the germline is not sequestered from somatic cells early in ontogeny and during the lifespan of the organism germ cells are continuously developing from somatic cells (Buss 1982; Blackstone and Jasker 2003; Müller et al. 2004; Seipel et al. 2004; Rinkevich et al. 2009; Rosner et al. 2009; Gold and Jacobs 2013; Dannenberg and Seaver 2018; DuBuc et al. 2020; Mueller and Rinkevich 2020; Vasquez-Kuntz et al. 2020). In non-chimeric metazoans, somatic and germ cell lineages share a single heritable genotype. In contrast, within a chimera, genotypically different somatic lineages compete for survival, as do germ cell lineages (Buss 1982; Stoner and Weissman 1996; Stoner et al. 1999; Rinkevich 2002a, 2002b, 2004a, 2004b, 2005a, 2011; Rinkevich and Yankelevich 2004; Simon-Blecher et al. 2004; Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). The genotype that dominates among the somatic cells likely confers some survival advantage and is subject to forces of natural selection. However, heritable germ cell lineages of one genotype may survive within the chimeric entity even though they do not contribute to the somatic tissue (Stoner and Weissman 1996; Stoner et al. 1999; Rinkevich and Yankelevich 2004; Laird et al. 2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). In these cases, the germline is said to hitchhike on or parasitize the soma of a different genotype, transferring heritable traits unseen by natural selection forces to subsequent generations that then express these non-selected "parasitic" traits. The newborn individual carrying a parasitic genotype need not reach sexual maturity to pass on an "unfit" germline genotype

to the next generation, as it may quickly fuse with adults or other offspring (e.g., Grosberg 1988) with "fit" somatic cells for continued germline hitchhiking. As a result, superparasitic germ cell genotypes, most capable of dominating foreign soma, may emerge in a population. Thus, germline parasitism may defy the Darwinian paradigm (Rinkevich 2011).

The above notions are further amplified in multichimerism (multipartner associations), where more than two allogeneic adult stem cells form a single botryllid ascidian colony (Rinkevich 1996; Rinkevich and Shapira 1999; Stoner et al. 1999; Paz and Rinkevich 2002). Multipartner chimeras grow faster and produce larger colonies when compared with chimeras made of two partners. They also exhibit other traits associated with more stable entities including fewer cases of morphological resorption or fragmentation events. Following the above, it was proposed that in multichimeras, the different intraspecific conflicts mitigate each other, generating an improved entity (the benefits of the conspecific adult stem cells living in a group exceed the cost of not doing so) where natural selection may act on the level of the whole colony instead of on each conspecific adult stem cell.

As in all stem cells, botryllid ascidian adult stem cells are self-renewing cells capable of differentiation. Five traits highlight these stem cells as genuine units of selection: (a) they efficiently migrate within the organism and between compatible organisms; (b) they compete with the host somatic and/or germline stem cells; (c) they express high and unlimited replication capacity; (d) they may share the soma with conspecific stem cells lineages and commonly determine specific traits for the benefit of the chimeric organism as a whole; (e) they can inhabit several different hosts. As a result, chimerism reflects cases where specific environmental pressures lead to the takeover of the fittest stem cells and their clones (Buss 1982; Rinkevich and Yankelevich 2004).

8. Future Directions on Stem Cells in Ascidians

Although ascidians represent a group of chordates exhibiting astonishing stem cell-mediated processes, most of the significant progress has been made in the last two decades. These advancements were mainly due to the application of unbiased methods translated from mammals to these marine invertebrates and the accessibility of omics methodologies. In the future, the study of ascidians will undoubtedly unravel stem cell potentialities, contributing to the basic knowledge of these cells. In this respect, ascidian simplicity and evolutionary closeness to vertebrates make them unique. Nonetheless, there are still several limitations to exploiting ascidians as a model organism for studies in stem cells biology, mainly due to the lack of methodological tools (such as cell lines, panels of specific monoclonal antibodies) and to the limitations of maintaining colonies in inland facilities, away from seawater supply.

8.1. Cell Cultures and Transgenesis

Immortal cell lines (established cell lines), including cell lines of adult stem cells, may provide an important tool in the research; yet, established cell lines for ascidians, as for all other marine invertebrates, are not yet available (Rinkevich 1999, 2005c). Nevertheless, several attempts in the last three decades have focused on the development of in vitro approaches. The first attempt (Rinkevich and Rabinowitz 1993) concentrated on the development of cell culture from the whole *B. schlosseri* hemocyte populations, followed by the establishment of embryo-derived cell cultures (Rinkevich and Rabinowitz 1994). Then, a series of studies followed the expression of stem cell-associated genes in in vitro cultures of epithelial cells from *B. schlosseri's* buds (Rinkevich and Rabinowitz 1997; Rabinowitz and Rinkevich 2004, 2005, 2011; Rabinowitz et al. 2009). Although well developed in solitary ascidians, transgenic lines are still not available for colonial species. This is mainly due to the difficulties encountered in treating eggs for gene delivery: in colonial ascidians, fertilization is internal, and eggs are enveloped by follicular cells. Yet, the availability of this technique, coupled with the transparency of colonial tissues, facilitates the ability to monitor in vivo the fate of stem cells and to uncover the molecular pathways that control stem cell proliferation and differentiation.

8.2. Monoclonal and Polyclonal Antibodies

Monoclonal antibodies (MAbs) are primary markers in biological sciences. The development of species-specific MAbs is highly valuable in research in general and in stem cells isolation. Initially, several MAbs were developed for experiments performed on *B. schlosseri*. The first sets were target antigens located on *B. schlosseri* hemocyte surface (Schlumpberger et al. 1984a) and embryonic cells (Schlumpberger et al. 1984b). An MAb that recognized all *B. schlosseri* hemocytes and zooids perivisceral epithelium was also developed (Lauzon et al. 1992). Aiming to develop MAbs that recognize epitopes involved in botryllid historecognition, Fagan and Weissman (1998) produced a MAb that labeled an epitope found on the atrial siphon and on the inner surfaces of hemolymphatic vessels. The above sets of experiments further revealed the existence of a MAb that specifically recognized and bound to all somatic cells of one genotype but did not react against somatic cells of another genotype and was used to follow somatic cell movements between partners within chimeras (Rinkevich 2004b). Ballarin et al. produced a monoclonal antibody recognizing a surface epitope on *B. schlosseri*'s germ and accessory cells, tunic cells, and hemocytes (Ballarin et al. 2011). However, the majority of previously tested MAbs are no longer available (Rinkevich personal communication), and renewed efforts are needed to establish new panels of MAbs for the research of stem cells in ascidians. In parallel, Lapidot et al. (2003) have established a MAb specific to the *B. schlosseri* pyloric gland cells, and Lapidot and Rinkevich (2005,

2006) developed panels of MAbs specific to cell surface antigens and to intracellular epitopes. Additional polyclonal antibodies were developed against specific stemness proteins of botryllid ascidians and include *B. schlosseri* specific anti-pl10, anti-vasa, anti-cadherin antibodies (Rosner et al. 2006, 2007, 2009), and *B. leachii* specific anti-piwi antibodies (Rinkevich et al. 2010).

8.3. Animal Breeding Methodologies

Established ex situ, inland culturing methods for ascidians species and the development of inbred lines and defined genetic stocks are important prerequisites for research (also alleviating seasonal availability of animals and laboratory acclimatization problems), primarily when dealing with stem cell studies. While research for the cultivation of ascidians under laboratory conditions started decades ago (e.g., Grave 1937), very little has been achieved when considering defined genetic stocks. For colonial species, animal breeding methodologies for the long-term development of inland brood stocks were employed primarily on *B. schlosseri*, representing three various ex situ approaches—one developed in Italy (Brunetti et al. 1984; Sabbadin 1960), another in the USA (Milkman 1967; Boyd et al. 1986) and Israel (Rinkevich and Shapira 1998), and a third, for *Botrylloides simodensis*, in Japan (Kawamura and Nakauchi 1986). Using classical breeding experiments, Yasunori Saito established defined homozygous and heterozygous lines for distinct histocompatibility genotypes (AA, BB, AB, and AX) that were crossed and maintained in the Hopkins Marine Station mariculture for several decades (De Tomaso et al. 1998; Voskoboynik et al. 2013b). These lines added compelling evidence that histocompatibility in *Botryllus* is controlled by a single gene, and they were used to isolate the *Botryllus* histocompatibility factor (BHF).

There were also attempts for inland culturing of other colonial ascidians, such as S. reptans (Sugino and Nakauchi 1987), and Didemnum vexillum (Fletcher and Forrest 2011; Rinkevich and Fidler 2014). Culturing systems have been established for four solitary species: *C. robusta*, *C. intestinalis*, *H. roretzi*, and recently, for *P. mytiligera* (Hendrickson et al. 2004; Joly et al. 2007; Li et al. 2020; Gordon et al. 2020).

8.4. Model Species for Studying Stem Cells and Ascidian Biodiversity

Most of the studies on regeneration and asexual reproduction in ascidians focused on a limited number of species, i.e., the solitary *C. intestinalis* and *C. robusta* and the colonial *B. schlosseri*. Several tools and protocols have been tuned for these animals and different laboratories use them as model species, even in absence of genetically defined lines. However, in recent years, molecular studies suggest the presence of cryptic species with the same nomenclature. In the case of *Ciona* sp., before 2015, the name *C. intestinalis* was used to indicate what is currently known as either *C. intestinalis* or *C. robusta* (Brunetti et al. 2015; Pennati et al. 2015; Gissi et al.

2017). Recently, the species *B. schlosseri* has been redescribed (Brunetti et al. 2017), since five divergent clades have been hypothesized under its name: *B. schlosseri* represents the clade A; *Botryllus gaiae* the clade E (Brunetti et al. 2020); clades B-C have not been determined yet. The uncertainty in species identification for botryllid ascidians has further been discussed (Reem et al. 2018). This equivocal taxonomical determination represents gaps of knowledge at several levels: firstly biological, but also operative, since methods, databases, and tools are developed in a laboratory and cannot be easily applied by other laboratories using different wild-type lines.

It is also worth noting that the ascidians exhibit an extraordinary variety of processes involving stem cells, and many of them are not manifested by *Ciona* sp. or *Botryllus* sp. For example, recently, extraordinary regenerative potentialities, going far beyond what is shown by *Ciona*, have been described in the solitary *P. mitiligera* (Gordon et al. 2021). Some *Botrylloides* species exhibit putative stem cell-based phenomena, such as torpor and constitutive WBR (Hyams et al. 2017; Kassmer et al. 2020) that are not exhibited by *B. schlosseri*. Future studies should consider these attributes.

8.5. Stem Cells and Immunity

The crosstalk between stem cells and immune cells during homeostasis and regeneration is well studied in mammals (Castillo et al. 2007; DelaRosa et al. 2012; Naik et al. 2018) but poorly investigated in aquatic invertebrates (Ballarin et al. 2021b). Studies on allograft rejections in ascidians point to potential relationships between stem and immune cells. For example, in the case of allograft rejections in the solitary ascidian *Styela plicata*, following the initial recruitment of cytotoxic morula cells to the graft area, an increase in the number of hemoblasts in the tunic surrounding the graft is observed (up to 30 days following rejection) (Parrinello 1996; Raftos et al. 1987). Similarly, in *Styela clava*, the injection of allogeneic hemocytes to the tunic induces the proliferation of hemoblasts within 5 days postinjection (Raftos and Cooper 1991).

Several events in the life cycle of colonial ascidians most likely involve interactions between stem and immune cells. These include (i) rejection of an allogeneic colony, (ii) chimerism, and (iii) resorption of zooids when the new generation buds replace them.

As described above, colonial ascidians exhibit natural stem-cell-mediated chimerism (Laird et al. 2005). When two genetically distinct colonies meet, they either anastomose extracorporeal blood vessels to form a chimera with a common vasculature or reject one another (Oka and Watanabe 1957b; Sabbadin 1962; Scofield et al. 1982; Voskoboynik et al. 2013b). In some chimeras, one of the chimeric partners undergoes partial or complete reabsorption (Rinkevich and Weissman 1992; Corey et al. 2016). Circulating germ and/or somatic stem cells of one partner in a chimera can compete with and replace the germline and/or soma of the other partner (Laird et al.

2005; Voskoboynik et al. 2008; Rinkevich et al. 2013). Therefore, stem cell engraftment in colonial ascidians is regulated on four different levels: (1) fusion or rejection; (2) if fusion occurs, the body of the losing partner is resorbed; (3) competition between circulating somatic stem cells to seed buds for asexual whole-body development; (4) stem cell competition among germline stem cells, which determines the genotype of the next generation.

Each level involves immune cell implications: the histocompatibility gene *BHF* controls fusion/rejection and limits stem cell parasitism to kin (Voskoboynik et al. 2013b); rejection is characterized by the extravasation of cytotoxic cells along the contact border and their degranulation and death with the consequent formation of points of rejection (Ballarin et al. 1995; Cima 2004; Rinkevich 2005b; Franchi and Ballarin 2017); resorption is a model for stem cell loss (failure to bud) when the immune system attacks the buds (Corey et al. 2016), and stem cell competitions relate to stem cell transplant engraftability.

The elimination of one partner in a chimera occurs mostly during a developmental period corresponding to a massive wave of programmed cell death and removal (Rinkevich and Weissman 1992; Cima et al. 2010; Corey et al. 2016; Franchi et al. 2016). Each blastogenic cycle in *B. schlosseri* ends in an apoptotic and phagocytic event of parental zooids, concurrent with the rapid development of next-generation primary buds (blastogenic "takeover" stage).

Using differential expression and gene set analysis, Corey et al. (2016) demonstrated that takeover pathways are co-opted by colonies to induce histocompatible partner elimination. These gene profiles show that colonies usurp developmental programs of autophagy, senescence, programmed cell death, and removal to eliminate allogeneic partners. This study also shows that the exposure of asexually propagating tissues to allogeneic cytotoxic and phagocytic populations has clear effects on development, leading to a developmental arrest. These findings suggest that the critical early events of asexual reproduction are dependent on protection from immune damage—a biological theme that emerges in higher vertebrates, where regulatory systems have evolved to create local sites of immune privilege such as for germ cell development or to protect a fetal allograft. The interactions between immune and stem cells in colonial ascidians are also suggested by a marked proliferative response observed following hemocyte xenotransplantation in *Botrylloides* (Simon-Blecher et al. 2004).

The life history of colonial ascidians, in which the interplay between stem and immune cells can be studied in vivo (Voskoboynik et al. 2008; Rinkevich et al. 2013; Corey et al. 2016; Rosental et al. 2018), offers an opportunity to better understand the relationship between immune function and regeneration.

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