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Review article

Cellular and molecular mechanisms of regeneration in colonial and solitary Ascidians

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A B S T R A C T

Regenerative ability is highly variable among the metazoans. While many invertebrate organisms are capable of complete regeneration of entire bodies and organs, whole-organ regeneration is limited to very few species in the vertebrate lineages. Tunicates, which are invertebrate chordates and the closest extant relatives of the vertebrates, show robust regenerative ability. Colonial ascidians of the family of the Styelidae, such as several species of *Botrylloides,* are able to regenerate entire new bodies from nothing but fragments of vasculature, and they are the only chordates that are capable of whole body regeneration. The cell types and signaling pathways involved in whole body regeneration are not well understood, but some evidence suggests that blood borne cells may play a role. Solitary ascidians such as *Ciona* can regenerate the oral siphon and their central nervous system, and stem cells located in the branchial sac are required for this regeneration. Here, we summarize the cellular and molecular mechanisms of tunicate regeneration that have been identified so far and discuss differences and similarities between these mechanisms in regenerating tunicate species.

1. Introduction

The ability of some animals to replace missing body parts has fascinated biologists for hundreds of years, and is widely distributed among the different metazoan phyla (Sanchez Alvarado, 2004). Many invertebrate organisms, including examples of the Hydrozoans, Anthozoans, Echinoderms, Annelids, Holothuroids Platyhelminthes and Tunicates, have robust regeneration capacity, yet only very few of these have been studied in detail. In contrast, most vertebrates have a very limited capacity to regenerate, except for some amphibians and teleosts that are able to replace appendages, tail, spinal cord, and heart.

Regeneration in many cases is dependent on the formation of a blastema; a mass of proliferating cells that give rise to newly differentiated cells to replace missing tissues. In vertebrates, epimorphic regeneration occurs by de-differentiation of lineage-restricted cells that subsequently acquire the ability to proliferate and give rise to differentiated progeny (Li et al., 2015). In many invertebrates, regeneration is dependent upon proliferation of pluripotent or multipotent stem cells that are present in the adult animal and capable of giving rise to a blastema.

Invertebrate chordates such as cephalochordates and tunicates have extensive regeneration capacities (Berrill, 1951; Somorjai et al., 2012). In colonial ascidians such as *Botrylloides leachii*, the entire body can regenerate from small fragments of extracorporeal vasculature, and these animals are the only chordates that are capable of whole body regeneration. Solitary ascidians such as *Ciona intestinalis* can regenerate distal structures upon injury, such as the cerebral ganglion and the oral siphon, so long as proximal structures are intact (Jeffery, 2015a, 2015b).

Regenerative processes involve the activation of many conserved developmental signaling pathways such as Wnt, Notch, TGF-beta and retinoic acid. Although these pathways are shared among all metazoan organisms, not all of these organisms are capable of regeneration. The question is how and in what specific cellular context are these pathways activated and regulated in regenerating species. During embryonic development, many transient pluri- and multipotent cell types are generated, but the persistence of these into adulthood differs between species, as does the ability of the more differentiated cell types to re-access these developmental pathways (Lai and Aboobaker, 2018).

It has been suggested that regeneration may be an ancestral chordate trait that has been lost in some lineages during vertebrate evolution (Oka, 1959), and recent studies suggest that highly conserved developmental signaling pathways, such as Notch, play important roles during regeneration in ascidians (Hamada et al., 2015; Zondag et al., 2016). Since tunicates are the closest living invertebrate relative to the vertebrates, the study of regeneration mechanisms in these animals will deepen our understanding of the evolution of regeneration across animal species and may to help advance biomedical approaches in the future.

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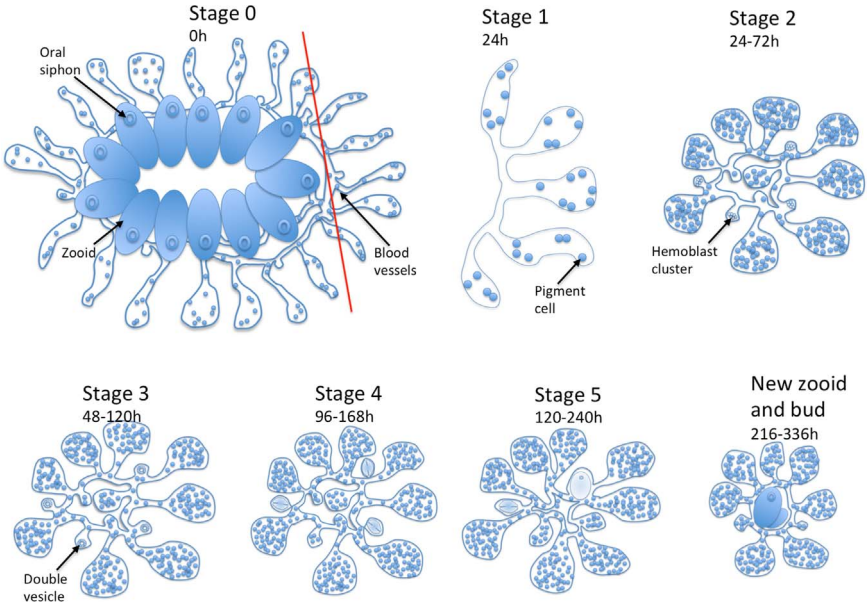

Fig. 1. Whole body regeneration from pieces of vasculature in Botrylloides. Genetically identical zooids share a common tunic and extracorporeal vasculature. When a piece of vasculature is cut away from the colony (stage 0), blood flow resumes within 24 hours (stage 1). Blood vessels undergo remodeling and become condensed and highly pigmented (Stage 2). Hemoblasts aggregate in niches formed by pockets of vascular epithelium. At stage 3, a double vesicle develops inside the regeneration niche. This tissue grows and undergoes invaginations (stage 4), followed by organ formation. At stage 5, the growing body has a heartbeat and opens the oral siphon. Once fully mature, the new body will begin palleal budding and give rise to a new colony.

Phylogenies based on both morphological and molecular data suggest that the Tunicates are monophyletic. Coloniality is found in different families of ascidians and appears to have evolved independently (Brown and Swalla, 2012). Many species of ascidians have remarkable regeneration capacities, however the cellular and molecular basis of regeneration has been studied in any detail only in colonial ascidians of the family Botryllidae, and in solitary ascidians of the Cionidae, and therefore this review is focused mainly on those species were those data are available.

2. Whole body regeneration in Colonial ascidians (Botryllidae)

Colonial ascidians of the family Botryllidae, such as various species of *Botrylloides* and *Botryllus,* are characterized by integrated colonies in which all zooids develop within a common tunic and share a vascular system with a constant blood flow (Fig. 1). Fig. 2 asexual reproduction in these species occurs by palleal bud formation, which ensues synchronously and is regulated by colony-wide developmental processes. During palleal budding, a new individual grows by formation of buds from a specialized epithelium in the adult (reviewed in Brown, 2012). Some species in the family of the Botryllidae have the striking ability to regenerate entire new bodies from small pieces of extracorporeal vasculature. This process, known as whole body regeneration (WBR), has been described mainly in different species of *Botrylloides*, such as *Botrylloides violaceus* and *Botrylloides leachii*, and appears to follow identical steps in these species. A staging system has only been established for *Botrylloides leachii* (Zondag et al., 2016), but based on the available literature, WBR follows the same basic pattern and stages in all species that are capable of it. Whether all Botryllidae have the capacity for whole body regeneration is currently under investigation. (Of note, no other part of the colony can regenerate a whole body, although developing palleal buds can continue development into fully functional zooid when separated from the adult.) During WBR, a tiny fragment of blood vessel that is separated from the rest of the colony is enough to give rise to a complete and functional new body within 7–14 days. The timing of WBR in *Botrylloides* is highly variable and depends on the species, the health of the colony at the time of injury, water quality and temperature, and possibly other factors. In Fig. 1, we provide a schematic of the morphological stages of regeneration with approximate timing.

After a piece of blood vessel is cut from the colony, blood flow via ampullar contractions (Blanchoud et al., 2017) resumes within 24 h. In the next few days, blood vessels undergo extensive remodeling, become rounded, condensed, and highly pigmented (Fig. 2). The terminal ampullae shrink, new vessel connections are formed, and the vascular system contracts into a dense network. Within this dense vascular tissue, an opaque mass of cells becomes apparent. In *Botrylloides violaceus,* Oka and Watanabe (Oka, 1959) observed the gathering of 15–20 small, undifferentiated cells (hemoblasts) under the epidermis of a blood vessel, followed by cell divisions that resulted in formation of a hollow blastula-like structure about 3 days following isolation of the blood vessel. The epidermis then continues to wrap itself around this vesicle, resembling the double vesicle stage commonly seen in palleal (asexual) budding (Fig. 1). Histological sections (Brown et al., 2009) show the development of an epithelial sphere surrounded by mesenchymal cells around 9 days post zooid removal. After the vesicle stage, a fold develops from the internal side of this sphere to become the endoderm, and regeneration begins to resemble the development of an asexual palleal bud. One report describes formation of a single-layered sphere of cells within the lumen of a blood vessel as early as two days after isolation from a colony (Rinkevich et al., 1995), and although multiple buds can initially form, only one will continue to develop into a zooid (Brown et al., 2009). Heart activity starts before the development of other organs is complete, and the fully mature zooid will begin filter feeding after ten days of regeneration. This new zooid then begins asexual reproduction to replace the entire colony (Oka, 1959).

In the closely related colonial ascidian *Botryllus schlosseri*, WBR requires a larger section of intact vasculature, and some reports suggest that it only occurs when surgical removal of bodies is performed during a specific stage of asexual reproduction, known as takeover, when zooids die and are replaced by palleal buds (Voskoboynik et al., 2007; Ricci et al., 2016).

Bodies form naturally from within blood vessels in some Botryllidae as part of their normal life cycle. This process is known as ‘vascular budding’ and is highly reminiscent of the steps observed in whole body regeneration of *Botrylloides*. In *Botryllus primigenus*, small, undiffer-

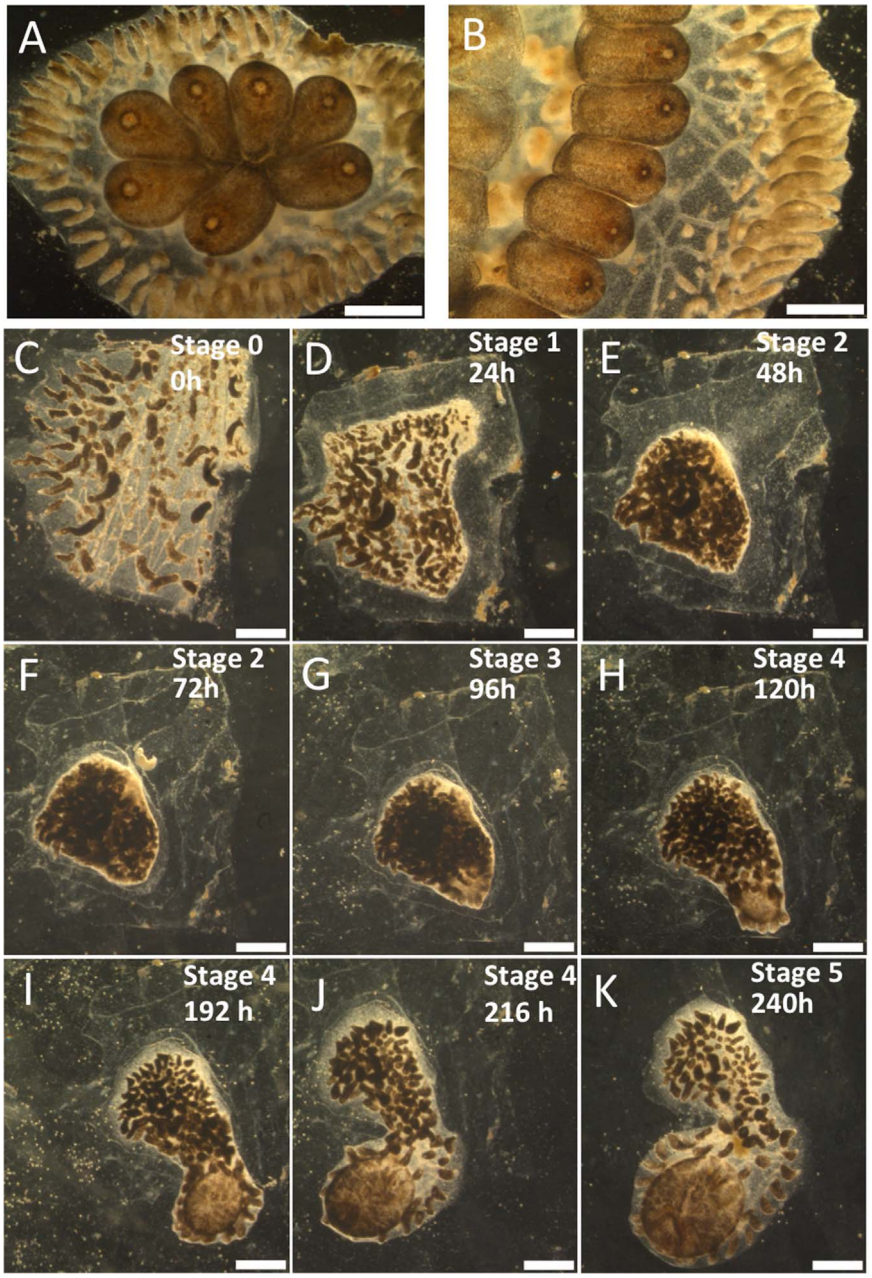

Fig. 2. Brightfield morphology of whole body regeneration in *Botrylloides leachii*. A: Brightfield image of whole *Botrylloides leachii* colony. B: Brightfield image of the extracorporeal vasculature of *Botrylloides leachii*. Scale bars in A and B: 1mm. C-K: Brightfield images of regeneration stages as outlined in Figure 1. C: Stage 0, blood vessel after surgical separation from colony. D: Stage 1. Within 24h, blood flow resumes and vessels begin to remodel. E and F: Stage2. Blood vessels remodel, become condensed and highly pigmented. G: Stage 3. A non-pigmented area (regeneration niche) becomes visible, containing a double vesicle. H -J: Stage 4. The regenerating tissue within the double vesicle grows and germ layers begin to differentiate, followed by organ formation. K: Stage 5. The regenerated body has developed a heartbeat and opens the oral siphon.

entiated hemoblasts aggregate in the blood vasculature and give rise to a solid mass that reorganizes into a hollow vesicle, then subsequently undergoes morphogenesis to develop a body (Oka, 1957). Vascular buds form in a similar fashion in a closely related species *Botryllus tuberatus* (Akhmadieva et al., 2007). In the closely related colonial ascidian *Symplegma brakenhielmi* (family Styelidae), zooids are embedded in a common tunic and surrounded by a communal vascular system in a similar fashion as in *Botrylloides*, but colonies appear less organized (Gutierrez and Brown, 2017). Formation of palleal and vascular buds occurs simultaneously and asynchronously. During *Symplegma* vascular budding, evagination of the vessel epithelium is observed, and small, round cells accumulate. The vesicle then expands to form a sphere, and an inner layer of cuboidal epithelium forms an inner vesicle before continuing organogenesis (Gutierrez and Brown, 2017). These observations suggest that whole body regeneration and vascular budding are highly similar processes that may share a common origin and common mechanisms. The only difference may be the signals that initiate the process: In the case of whole body regeneration, separation of the vessels from the colony is required to trigger formation of bodies from vessels, whereas in species with vascular budding, injury is not required and the signal is always "on". Oka and Watanabe (Oka, 1959) suggested that vascular budding is a primitive and ancestral type of budding that has been suppressed in some colonial ascidians in favor of palleal budding. Separation of blood vessels from the rest of the colony may artificially remove this suppression and trigger vascular budding.

Evidence for this idea comes from recent study showing that even in *Botrylloides leachii,* vascular budding can occur without injury, and is

induced by stressors such as decreased temperatures and unfavorable conditions (Hyams et al., 2017). Under such circumstances, the colony can enter a hibernation stage (termed: Torpor), where all bodies are resorbed and only a condensed aggregate of blood vessels remain. This dormant state can last for months, however; once conditions improve, a new body will regenerate from within the blood vessels and give rise to a new colony. Morphologically, this development of bodies from hibernating vessels is very similar to whole body regeneration induced by injury. In the wild, a colony may recover from various factors such as unfavorable environmental conditions or injury from a predator in this way.

In addition, budding behavior may be a mode of adaptation to environmental conditions, as it can differ between populations of the same species isolated from different regions, as seen in *Smyplegma brakenhielmi*. Colonies from Panama only undergo vascular budding, whereas colonies from Brazil undergo both palleal and vascular budding (Gutierrez and Brown, 2017). This suggests either an environmental influence on vascular budding, or intrinsic genetic diversity between local populations, thus caution must be taken when comparing results from samples of the same species collected in different parts of the world. Maintaining genetically inbred strains that are shared between laboratories would be ideal, however; this is complicated by the low fitness of, for example, inbred *Botryllus schlosseri* colonies. Other colonial ascidians are very difficult to propagate in the laboratory and need to be collected from the wild, therefore more effort is needed to overcome these limitations in the future.

2.1. Cellular origin of whole body regeneration

To date, the cell types that give rise to the tissues that are formed during whole body regeneration or vascular budding have not been clearly identified. It remains to be determined if newly formed somatic tissues arise from a population of stem cells, whether these stem cells are pluripotent or have lineage restricted potential, and whether de-differentiation plays a role. Morphological observations suggest that vascular buds in *Botrylloides, Botryllus primigenus, Botryllus tubaratus* and *Symplegma* originate from an undifferentiated population of blood borne cells. In colonial ascidians, small, undifferentiated cells in the blood or coelomic fluid have been termed “hemoblasts” (Oka, 1957; Kawamura and Sunanaga, 2010). These cells appear undifferentiated, have a transparent cytoplasm and no nucleolus, and have been implicated in contributing to somatic tissues during asexual budding, whole body regeneration, and vascular budding (Kawamura and Sunanaga, 2010). An increase in the population of hemoblasts is observed 15 h post injury during whole body regeneration in *Botrylloides leachii* (Blanchoud et al., 2017).

During whole body regeneration in *Botrylloides violaceus*, Piwi expression is seen in circulating hemoblasts temporally near the early vesicle stage and occasionally within the epithelium of a vesicle (Brown et al., 2009). This suggests that Piwi-positive hemoblasts integrate into the vesicle. During stages of organogenesis, many Piwi-positive cells are found adjacent to tissue layers, with occasional presence within a layer (Brown et al., 2009), and siRNA mediated knockdown of Piwi-expression inhibits whole body regeneration (Rinkevich et al., 2010). These data suggest that hemoblasts contribute to somatic tissues during whole body regeneration.

In colonial ascidians, mobile germline stem cells expressing *vasa* are present in the blood and give rise to gonads during asexual reproduction (Laird et al., 2005; Kassmer et al., 2015; Kawamura and Sunanaga, 2011; Brown and Swalla, 2007). Piwi, like *vasa*, is also expressed in blood borne germline progenitors (Brown and Swalla, 2007; Sunanaga et al., 2010). Piwi and vasa belong to a group of genes that are normally associated with the germline. Interestingly, in some invertebrates these same genes can be expressed in multipotent cells that are not fully germline restricted, and thus have somatic potential (Juliano et al., 2010). In some animals, an irreversible separation of germline and soma occurs early in embryonic development, but many adult flatworms, cnidarians, and sponges contain multipotent or totipotent stem cells that give rise to various adult cell types, including the germ line (reviewed in (Juliano et al., 2010)). The function of these cells depends on *piwi*, *vasa*, *tudor*, and *pumilio* - genes that were first identified in germline stem cells. Therefore, in ascidian species that are able to undergo whole body regeneration, the vasa/piwi-positive population of hemoblasts may contain both germline- and somatic progenitors, and there may be a primitive population of cells not restricted to either germline nor soma. In single cell transplants between genetically distinct *B. schlosseri* colonies, germline and somatic engraftment never came from the same cell, suggesting that separate lineages of somatic and germline stem cells exist, at least in this particular species (Laird et al., 2005). More markers need to be identified to discriminate between potential somatic stem cells, germline stem cells, and pluripotent stem cells that may exists within the “hemoblast” population amongst different *Botryllid* species.

In colonial ascidians that undergo vascular budding as part of their normal life cycle, blood borne stem cells may naturally have a wide differentiation potential. When vascular budding is suppressed under normal conditions, like in *Botrylloides*, these stem cells may be dormant before activating upon stress or injury. Future studies will reveal what those suppressing or activating signals are. It is also possible that the differentiation potential of cells within the vasculature may be induced to change upon injury. When no bodies and buds are left, de-differentiation of mobile or non-mobile cells may give rise to progenitors that lead to the formation of new bodies. De-differentiation is the basis for limb regeneration in vertebrates and is a very effective means of producing new tissue from pre-existing cells (Li et al., 2015). Whether de-differentiation is involved in either whole body regeneration or vascular budding is currently unknown.

It is evident that the vasculature plays an important part in whole body regeneration and vascular budding, but its exact role has not been fully characterized. Upon separation from the rest of the colony, the remaining blood vessels undergo extensive remodeling and re-shaping, eventually forming pockets or niches where hemoblasts aggregate (Fig. 1). How and why this remodeling occurs and how it leads to the formation of niches has not been investigated, though it has been suggested that the epithelium that lines the blood vessels can develop into the ectoderm of vascular buds (Oka, 1957) by giving rise to the outer wall of the double vesicle. A double walled vesicle is also formed during palleal budding, with the outer wall derived from the epidermis of the parent zooid, and the inner wall, which gives rise to the organs, from the peribranchial (atrial) epithelium of the parent zooid. The gut primordium originates from the atrial epithelium. Hemoblasts migrate into the space between the two layers of the double vesicle – their contribution to palleal bud formation is not well understood, but genetic tracing in *Botryllus schlosseri* confirmed that at least the intestine of a palleal bud does not originate from a mobile progenitor (Carpenter et al., 2011). Oka and Watanabe (Oka, 1957) describe vascular budding in *B. primigenus* as mesoblastic, wherein mesenchymal cells circulating in the blood stream form the inner wall of the vesicle. If this is true, then the very first steps of palleal budding and vascular budding occur by very different mechanisms and from different cellular sources, however; both lead to the formation of a double vesicle. After that, bud development in both cases is strikingly similar (Ricci et al., 2016).

Some evidence for a contribution of mobile hemoblasts to somatic tissues comes from other colonial ascidians. *Perophora viridis* undergoes stolonial budding. Colonies irradiated with X-rays do not form stolonial buds, but bud formation resumes upon injection of coelomic hemoblasts (Freeman, 1964). In *Symplegma reptans*, detailed electron micrographs strongly suggest that hemoblasts develop into body muscle cells around the developing siphon during palleal budding. Myofilaments appear in the cytoplasm of hemoblasts that aggregate around the epidermis of the oral siphon (Sugino et al., 2007).

In the future, lineage-tracing experiments will hopefully provide insight into the differentiation potential of different hemoblast populations. Efforts are currently underway to generate transgenic colonial ascidians which will enable more precise cell tracking, gene manipulation, and help provide a more detailed analysis of the cellular sources underlying whole body regeneration and vascular budding. Current resources for colonial ascidians include: Published genomes for *Botrylloides leachii* and *Botryllus schlosseri* (Voskoboynik et al., 2013; Blanchoud et al., 2018) mRNA seq and transcriptome database (Rodriguez et al., 2014) Fluorescent in situ hybridization (Langenbacher et al., 2015), qPCR, small molecule inhibitors (Kassmer et al., 2015). sSiRNA –mediated gene knockdown has been published by several labs using different approaches, and so far there appears to be no consensus on the proper technique for this approach (Rinkevich et al., 2010; Sunanaga et al., 2010; Tiozzo and Tomaso, 2009). The success of this technique appears to be highly dependent on the particular gene and context. Microinjection of embryos is currently being attempted, and if successful will hopefully generate CRISPR mutants and transgenic lines soon.

3. Pathways involved in whole body regeneration

A large-scale gene expression study identified a few pathways that are upregulated during whole body regeneration in *Botrylloides leachii* (Zondag et al., 2016). During the first 24 h after injury, some highly conserved pathways that have previously been shown to be involved in development and regeneration in other chordates, such as Wnt, Notch, TGFbeta and Hedgehog, are upregulated. TGFbeta is required during axolotl limb and xenopus tail regeneration, and the Wnt pathway is involved in regenerative processes in many species, like zebrafish, planarians and axolotl (Li et al., 2015). During the early stages of whole body regeneration, cell-cell and cell-matrix-adhesion as well as metabolic pathways and apoptotic pathways are upregulated. During later stages, translational processes as well as cellular component biogenesis are upregulated, such as 58 genes in the ribosomal pathway – consistent with increased synthesis of proteins. Wnt, Notch and TGFbeta are down regulated in later stages of regeneration (Zondag et al., 2016). Their expression may only be transiently required during the early stages of regeneration. The wnt pathway is know to play important roles in morphogenesis during asexual reproduction (Di Maio et al., 2015), but its function during the early stages of whole body regeneration has not yet been characterized.

Retinoic acid signaling controls cellular differentiation during embryonic development and controls the formation of the blastema during zebrafish fin regeneration (Tal et al., 2010). During whole body regeneration in *Botrylloides leachii*, retinoic acid receptor is expressed in aggregates of hemocytes, and retinoic acid synthesis inhibitors block regeneration (Rinkevich et al., 2007).

Transcription factors associated with germ layer specification during embryogenesis, such as FoxA1, GATAa, GATAb, Otx, Gsc and Tbx2/3 are expressed in developing vascular buds in a similar fashion as during asexual reproduction (Ricci et al., 2016).

These results suggest that whole body regeneration in colonial ascidians is controlled by a similar set of pathways as regenerative processes in other animals. The exact functions of these pathways during these complex processes leading to the formation of an entire new body remain to be investigated.

4. Regeneration from multipotent epithelia in colonial ascidians

Colonial ascidians contain multipotent epithelial tissues such as the epicardium, septum, and atrial epithelium (reviewed in Kawamura et al. (2008)). These tissues normally give rise to buds during asexual reproduction but can also replace missing body parts in the adult animal upon injury.

One example is *Polyandrocarpa misakensis*. When cut in half, the anterior part of the adult zooid does not contain the posterior components such as the esophagus, stomach and intestine, however; it regenerates these organs within a week, and they are formed from the atrial epithelium near the cut surface (Kawamura et al., 2008; Kaneko et al., 2010). Within the first 48 h, the atrial epithelium invaginates into the mesenchyme space and gives rise to the gut primordium. The invaginating epithelial cells change their appearance to cuboidal, while the other parts of the atrial epithelium remain squamous. The stomach and intestine differentiated 3–4 days after the amputation, and inhibitors of retinoic acid (RA) synthesis suppressed regeneration and gut formation. Addition of 13-*cis* RA rescues regeneration, further suggesting that RA is required for the regeneration of the gut (Kaneko et al., 2010).

A recent study investigated the mechanisms of de-differentiation of the atrial epithelium during regeneration and found a correlation between de-differentiation and autophagy (Kawamura et al., 2018). Ultrastructural observations show that the numbers of autophagosomes, lysosomes, and secondary lysosomes increase remarkably, concomitant with mitochondrial degradation and exosome discharge. Autophagy had been previously shown to play roles in regeneration blastemas of planarians (Gonzalez-Estevez et al., 2007) and zebrafish (Varga et al., 2014).

In *Clavelinidae*, when the thorax containing the pharynx, brain, and siphons is surgically removed from the abdomen, a new thorax is regenerated from the multipotent epicardium (Kawamura et al., 2008; Brien, 1932, 1968). It will be interesting to investigate whether all tunicate species that contain multipotent epithelia are able to regenerate adult body parts.

5. Regeneration of distal body parts in Ciona

In solitary ascidians such as *Ciona intestinalis*, regeneration capacities are more restricted than in colonial species. These animals only reproduce sexually, and are capable of partial body regeneration, in which only some parts of the body can regenerate the missing parts of an animal. Specifically, distal body parts can be replaced from proximal parts, but only if a portion of the branchial sac (Fig. 3) is present, and distal parts are unable to regenerate the proximal parts. Even after the most basal bisection, only the basal parts that contain the branchial sac will regenerate the distal parts, and middle portions of trisected animals containing the branchial sac will regenerate only the distal parts (Jeffery, 2015a). *Ciona* is one of the largest of all ascidians, regenerative experiments comparatively easy to perform. Along its proximal (base) to distal axis it contains the visceral organs (heart, gonad, stomach, and intestines), the pharynx containing a large branchial sac, the neural complex, and the oral and atrial siphons (Fig. 3). The oral siphon is composed of muscular tissue, nerves, vasculature, epidermis and eight oral pigment organs (OPO) - sensory

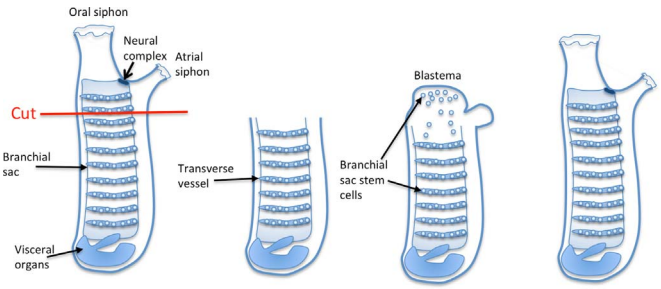

Fig. 3. Siphon regeneration in Ciona. Surgical bisection removes distal structures such as the oral siphon and the neural complex. The proximal parts contain the branchial sac and visceral organs. Cells within the transverse vessels of the branchial sac begin proliferating. A blastema forms at the cut site, containing potential stem cells that have migrated from the branchial sac. The blastema differentiates into a new oral siphon and neural complex.

receptors rimming the siphon opening. The oral siphon can regenerate within a month after removal. During regeneration of the oral siphon, a blastema of proliferating cells is formed about 4 days after injury. Within 10 days, the OPO and circular muscle fibers are regenerated, before the new oral siphon grows to full length. Interestingly, a large wound in one of the siphons causes the creation of a new siphon tube in the wounded area, and the same phenomenon was discovered in another solitary ascidian, *Styela clava* Jeffery (2015). All he distal tissues of the ectopic siphon were formed in the correct proportions. Ciona lives for about 1 year and grows continuously until death, but regeneration capacity declines with age, and regrowth of the oral siphon becomes progressively slower during aging (Jeffery, 2012).

5.1. Role of stem cells in oral siphon regeneration

Elegant experiments by W. Jeffrey show that cells from the branchial sac are involved in distal regeneration of the oral siphon. As mentioned above, the middle portions of trisected animals, which contain the branchial sac, can also regenerate the distal parts (Jeffery, 2015a). As soon as 3 h after injury, dividing cells within the transverse vessel of the branchial sac incorporate the nucleoside analog Edu (5-ethynyl-2′-deoxyuridine), and a large number of labeled cells can be seen in the lymph nodes. These same cells also label with alkaline phosphatase (AP) and an anti-piwi antibody, suggesting that they are undifferentiated, and possibly stem cells. High AP activity is traditional marker of pluripotency in mouse and human stem cells (Stefkova et al., 2015). At this early stage of regeneration, none of the cells in the regenerating oral siphon incorporate Edu, however; after a 24 h labeling period with Edu, and a 10-day chase, Edu positive cells appear in the regeneration blastema at the oral siphon. This suggests that branchial sac stem cells migrate to the oral siphon regeneration blastema. In transplantation experiments, Edu-labeled branchial sacs were removed, stained with neutral red and transplanted into the branchial sac of a host animal. After bisection, Edu positive cells were detected in the regenerating oral siphon of the host. In older animals that have reduced regeneration capacity, AP and piwi staining is weaker, suggesting declining numbers of branchial sac stem cells. These data strongly suggest that cells within the branchial sac that are recruited into cell cycle upon injury are stem cells that can give rise to regenerating structures within the oral siphon. Longer cell tracking experiments will be needed to assess incorporation of their progeny into differentiated tissues of the regenerated oral siphon, such as the cerebral ganglion, and single cell transplantation would clarify if they are multipotent at the single cell level. To assess the source of the cells replacing the OPO, the remaining siphon stump that was left after OS amputation at the distal tip was cultured as an explant. New OPO were regenerated in the distal region of the explants, indicating that local cells give rise to the new OPO (Auger et al., 2010).

5.2. Pathways regulating oral siphon regeneration

Gene expression analysis of regenerating oral siphons by micro-array showed that members of the notch-signaling pathway are upregulated during regeneration. These include the ligands delta and jagged, and the notch receptor. Expression of delta1, notch and hes-b were detected in regenerating tissue, including the blastema (Hamada et al., 2015). Notch was also expressed in the lymph nodes along the transverse vessel of the branchial sac, where potential stem cells are located. Chemical inhibition of notch signaling blocked siphon regeneration and prevented proliferation of branchial sac stem cells. Notch signaling is involved in amphibian tail regeneration (Beck et al., 2003) and is required for blastema cell proliferation during teleost fin regeneration (Grotek et al., 2013; Munch et al., 2013).

Another study used RNA-seq to identify 472 mRNAs that are differentially expressed during oral siphon regeneration. Among them are apoptosis related genes, proliferation related genes, TGF-beta pathway activators and transcription factors (Spina et al., 2017). 23 micro-RNAs were also differentially expressed. Categories of biological processes upregulated in the early stages of regeneration included wound healing, stress response, activation of morphogenetic processes and signaling. At day 3, wnt and hedgehog signaling related genes are transiently upregulated. TGF-beta activators were upregulated post-amputation, and treatment with an inhibitor of TGF-beta signal transduction completely blocked regeneration.

Again, these results emphasize the idea that regeneration in ascidians is controlled by conserved pathways that are also involved in regeneration in animals from other phyla.

5.3. Central nervous system regeneration in Ciona

In addition to being able to regenerate oral siphons, Ciona is capable of central nervous system regeneration. The nervous system of tunicates consists of the cerebral ganglion, nerves within the body wall, the visceral nerve, the dorsal strand plexus and sensory structures. The cerebral ganglion contains most of the neurons of the central nervous system (CNS), and nerves extend from the ganglion to the siphons, body wall and caudal viscera. The neural complex (NC), which contains the central ganglion (brain), the neural gland, and the ciliated duct and funnel, can regenerate after removal of the distal part of the body (REF Mingazzinin 1891).

After removal of the neural complex, including part of the pharynx and the body wall, the neural complex regenerates completely within about a month (Dahlberg et al., 2009). The cellular origin of the regenerated neural complex is poorly understood and likely involves more than one cell type. GnRN positive neuroblasts from the dorsal strand are initially detected near the regenerating cerebral ganglion after NC ablation, and subsequently develop axonal processes and become incorporated into the posterior portion of the regenerating ganglion. Dahlberg et al. reported that new structures originated from the residual neural structures on all sides of the ablation area. At the tip of each nerve, incorporation of the proliferation marker Edu indicated the formation of a blastema which gives rise to new nerve cells. The neural complex regulates the contractile behaviors of the muscular body wall and siphons, including the so-called “cross-siphon” reflex (Mackie and Wyeth, 2000; Mackie et al., 2006). These behaviors are gradually restored after ablation as connections are re-formed between nerve tracts of the CNS and the regenerating ganglion (Dahlberg et al., 2009).

5.4. Regeneration in other solitary ascidians

Polycarpa mytiligera can regenerate its gut and branchial sac. Following gentle squeezing of the tunic these animals can eviscerate a large portion of their gut through the oral siphon (Shenkar and Gordon, 2015). The disemboweled part of the digestive tract is usually composed of the gut loop, including the stomach, and a large endocarp (a projection of the body wall into the atrial cavity) attached to the gut. A torn branchial sac, an esophagus and rectum are left behind in the animal. Twelve days after evisceration, animals had regenerated a completely new gut with fecal pellets and a mucus thread in the branchial sac, implying active filter-feeding (Shenkar and Gordon, 2015).

6. Conclusions

Undifferentiated cells that are likely multipotent or pluripotent stem cells seem to be involved in both whole body regeneration in colonial ascidians as well as in distal regeneration in solitary ascidians. In *Botrylloides leachii*, undifferentiated hemoblasts that express piwi appear to be involved in regeneration, and in *Ciona*, piwi-positive stem cells from the branchial sac are required for siphon regeneration. These cells are mobile, appear to be maintained as a pool of undifferentiated cells and proliferate in response to injury. In *Ciona*, it has been

demonstrated that branchial sac stem cells give rise to regenerating tissues. In *Botrylloides*, this question remains to be investigated. Likewise, it remains to be formally tested whether these undifferentiated cells are self-renewing stem cells or whether they potentially arise from de-differentiation. Likewise, it will be important to test whether these putative stem cells lineage restricted, multipotent or pluripotent, and whether they overlap with the population of mobile germline stem cells. Germline stem cells in colonial ascidians express piwi (REF), and therefore, it is possible to speculate that a population of totipotent stem cells with germline and somatic potential could exist in these animals.

Whole body regeneration and distal regeneration share similar set of signaling pathways and molecular mechanisms that are activated upon injury. These include Notch, wnt, TGFbeta and hedgehog. All of these pathways have conserved roles in regeneration across phyla (Sanchez Alvarado and Tsonis, 2006), where they are required for the proliferation of blastema cells, or are involved in regulating differentiation. Some striking cross-species similarities in the functions of these pathways during regeneration are beginning to be uncovered. In Zebrafish, Notch regulates blastema cell proliferation in zebrafish (Munch et al., 2013) and plays a similar role in *Ciona*, where inhibition of Notch reduces proliferation of branchial sac stem cells (Hamada et al., 2015). The roles of these pathways have not yet been experimentally tested during whole body regeneration, but it is likely that the roles of some of these molecular pathways are shared between different modes of regeneration in tunicates.

While regeneration in some tunicates appears to be linked to the presence of undifferentiated cell types, de-differentiation seems to play a role in other cases. In *Polyandrocarpa misakensis,* the atrial epithelium that had been classified as multipotent shows clear ultrastructural signs of de-differentiation prior to giving rise to new organs (Kawamura et al., 2008; Kaneko et al., 2010). Once appropriate tools, such as transgenics, become available, future studies will aim assess whether multipotent epithelial tissues found in colonial ascidians, such as the epicardium, septum, and atrial epithelium, are truly multipotent, and whether or not they undergo de-differentiation during asexual reproduction and regeneration.

Some theories suggest that regenerative capacity is linked to the amount of resources invested into the adult body, but because of the high cost of this process, it is selected against if investment in reproduction increases fitness of the species (Lai and Aboobaker, 2018). In colonial ascidians, bodies are transient structures that are required for sexual reproduction and feeding, whereas the vasculature is permanent. The bodies carry the gonads that are replaced every few days by asexual reproduction. The ability for whole body regeneration from the vasculature may have been selected for because it is vital for these organisms to be able to replace lost bodies in order to continue sexual reproduction.

Within the chordates, the range of regenerative capacities is very broad and ranges from wound healing associated with scarring (humans, mice) to the capacity of fully regenerating limbs and spinal cords without scars (amphibians) to whole central nervous system regeneration (*Ciona*), and, finally, to the extreme case of whole body regeneration (*Botrylloides*). Because of their phylogenetic position as invertebrate chordates, the study of regeneration in tunicates is vital to understanding the evolution of this wide range of regenerative capacities within the chordates. Tunicates seem to be the only chordates that have undifferentiated, multipotent stem cells that are involved in regeneration. In all other cases of regeneration in chordates (axolotl limbs and spinal chord, zebrafish fins and hearts), the new tissues arise from terminally differentiated cells that re-acquire proliferation capacities and de-differentiate within their pre-defined lineage. Future studies will investigate the cellular and molecular mechanisms underlying the regenerative processes in our close relatives, the tunicates, and will be crucial for the advancement of human regenerative medicine.

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