EvoRegen in Animals: Time to uncover deep conservation or convergence of adult stem cell evolution and regenerative processes

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Abstract

How do animals regenerate specialised tissues or their entire body after a traumatic injury, how has this ability evolved and what are the genetic and cellular components underpinning this remarkable feat? While some progress has been made in understanding mechanisms, relatively little is known about the evolution of regenerative ability. Which elements of regeneration are due to lineage specific evolutionary novelties or have deeply conserved roots within the Metazoa remains an open question. The renaissance in regeneration research, fuelled by the development of modern functional and comparative genomics, now enable us to gain a detailed understanding of both the mechanisms and evolutionary forces underpinning regeneration in diverse animal phyla. Here we review existing and emerging model systems, with the focus on invertebrates, for studying regeneration. We summarize findings across these taxa that tell us something about the evolution of adult stem cell types that fuel regeneration and the growing evidence that many highly regenerative animals harbor adult stem cells with a gene expression profile that overlaps with germline stem cells. We propose a framework in which regenerative ability broadly evolves through changes in the extent to which stem cells generated through embryogenesis are maintained into the adult life history.

Keywords

Regeneration, evolution, invertebrates, neoblasts, stem cells, piwi

Introduction

The study of regenerative processes in animals now promises to reveal mechanisms and processes that will generate new and important biomedical knowledge, but also offers the opportunity to study the evolutionary biology of regeneration across animals (Bely and Nyberg 2010; Tanaka and Reddien 2011). An inclusive definition of regeneration we use is 'the ability of adult cells to use some combination of proliferation, migration and differentiation for the purpose of ensuring continued biological function in adult animals'. However, the extent of regenerative ability defined in this way is highly variable between and within different phyla (Bely and Nyberg 2010). This is not surprising given that regenerative capacity reflects the extent of resource investment into the maintenance of the adult organism, and this will be different in each species and within different tissues and organs. So, while ostensibly the ability to extensively regenerate adult body structures would seem like an advantage all animals would benefit from, the cost of this process means that it will be selected against whenever the investment of resources elsewhere, normally into reproduction, increases overall fitness (Bely and Nyberg 2010; Grillo et al. 2016).

This leaves us asking many exciting questions about exactly how and why some animals are capable of extreme regenerative feats like whole body regeneration (WBR), and others are not. For example, how do highly regenerative states, making use of both the deeply conserved components of metazoan genomes (e.g. *Wnt* signalling) but also novel lineage specific genes, evolve from less regenerative states? What are the selective pressures that maintain extravagant (and costly) regenerative feats (e.g. a dependence on asexual reproduction, the diversion of predators through autotomy, response to infection) or is enhanced regenerative ability in fact partly an emergent property of another evolutionary process (e.g. heterochronic shifts in life history)? To what extent in different taxa does regeneration recapitulate developmental mechanisms? How does regenerative capacity change within specific tissues and organs in an organism, and what evolutionary factors drive these changes? The broader (phylogenetic) and deeper (mechanistic) understanding of developmental processes, and established approaches and frameworks put in place by very successful studies of the evolution of developmental processes (EvoDevo), as well as greater experimental tractability of model systems means previously intractable questions like these can now be tackled within the context of a comparative evolutionary study of regeneration.

Similar to EvoDevo, it is only through comparative study of regeneration (or EvoRegen for short) in a number of different regenerating animal groups that allow comparisons over short and

long phylogenetic distances that these questions will be answered. As a step towards creating a framework for tacking questions in EvoRegen it seems timely to outline what we know about the evolution of adult stem cells (or other cells that might not be classed as stem cells) that drive regeneration across animals.

An overview of highly regenerative animal models amongst invertebrate phyla

Reconstructing the evolution of regenerative ability requires comparisons of regeneration across divergent taxa. Here, we focus on the invertebrate phyla as these include all the taxa capable of the most extreme examples of WBR. Additionally, experimental manipulations of adult invertebrates to study regeneration, with the notable exception of Cephalopoda, do not have the same ethical restrictions that are placed on vertebrate taxa. As invertebrates from deuterostome (Tunicata, Echinodermata), protostome (Spiralia, Ecdysozoa) and basal branching bilaterian (Acoelomorpha) and non-bilaterian (Cnidaria, Porifera) clades all offer tractable models for functional studies of regeneration, the evolution of regenerative mechanisms can now be compared across the whole of Metazoa. As these lineages are interspersed with species with relatively limited regenerative capacity we know that regenerative ability has increased and decreased multiple times during the radiation of metazoan phyla (Bely 2010). For instance in the model nematode *Caenorhabditis elegans,* no regeneration events fitting the definition we suggest above are observed, but even here cell level regeneration is observed as axons are capable of regrowth after injury (Yanik et al. 2004; Yanik et al. 2006; Chen et al. 2011). This does not involve cell division and the extent of differentiated neuron regrowth after injury exhibits considerable variation, dependent upon developmental stage, neuron type and the position of axotomy (Wu et al. 2007; Ghosh-Roy and Chisholm 2010). Currently a number of different animals have been used in regenerative studies, and are presently at different stages of development with respect to available tools and what we know about their regenerative capacities and underlying cell biology (Figure 1, Figure 2). Clearly for all these groups further development is required, but the rapid progress being made means that this is the most exciting time in history to be studying regenerative biology.

One feature of the most (but not all) regenerative animals seems to the presence of potentially pluri- and multi-potent stem cells that share similarity with extensively studied germline stem cells (GSCs) that give rise to the germline in *Drosophila*, *C. elegans* and mice (Juliano et al. 2010; Solana 2013; van Wolfswinkel 2014; Gehrke and Srivastava 2016). Specifically, all these

cells contain RNA/protein rich structures referred to as nuage, germ plasm or chromatoid bodies (nuGPCB) that often contain protein products of germline associated genes like *vasa*, *nanos*, *piwi*, *tudor*, *pumilio* and *bruno* family genes (Figure 2). These electron-dense perinuclear structures are found in many different organisms, e.g. chromatoid bodies in planarians (Auladell et al. 1993; Sato et al. 2001; Solana et al. 2009; Rouhana et al. 2012; Rouhana et al. 2014), P granules in *C. elegans* (Gruidl et al. 1996; Pitt et al. 2000), polar granules in fruit flies (Hay et al. 1988; Hay et al. 1990; Amikura et al. 2001) and Balbiani bodies in arthropods (Kloc et al. 2014), mice oocytes (Pepling et al. 2007) and many other animal taxa (Kloc et al. 2004). Several genetic components of nuGPCB are also expressed in somatic cells such as pluripotent or multipotent cells, and this has led to the hypothesis of the existence of a Germline Multipotency Program (GMP) that is shared between the germline and these adults stem cells (Juliano et al. 2010). The most accepted shared characteristic of these cells is the expression of PIWI/piRNAs that are thought to help ensure genome stability by silencing transposons, with some of the other nuGPCB components also thought to involved in this regulation (Weick and Miska 2014). In summary, we can say that there is strong (but not complete) correlation between the presence of expression of GMP components in somatic stem cells and the extent of regenerative capacity and the ability to perform WBR.

One pertinent question that should now be tractable is whether these pluri- and multipotent adult stem cells have a common single origin from the germline in Metazoa or represent a series of independent derivations (Figure 3). Recently, Solana (Solana 2013) couched this problem in terms of breaking the classical Weismann Barrier (WB) that was postulated to keep the totipotent immortal germline distinct from the soma, ensuring information only went from germ line to soma. Solana hypothesized that this event allows so called “primordial stem cells” that contain germ plasm and have the role of ensuring germline continuity, too also contribute to somatic tissues, where their presence underpins the evolution of WBR and asexual reproduction by fission. This breaking of the WB between germline and soma may have occurred ancestrally once with re-establishment in some lineages, or multiple times independently in different lineages. Alternatively, this barrier may not have existed ancestrally and could have been formed independently in different lineages.

Another way to look how regenerative ability evolves, that would include all levels of regenerative capacity, is in terms of the evolutionary changes in the cell lineages and regulatory pathways that persist in adults after development. From a totipotent zygote, many intermediate pluri- and multipotent cell states are generated but the extent to which these lineages persist in

adults varies greatly as does the capacity of remaining cells to (re)access developmental regulatory pathways. This seems to be a major factor in deciding the extent of regenerative ability. Evolutionary changes to which stem cells (if any) are maintained and which regulatory pathways in different cells and tissues remain accessible in adulthood would then directly influence the extent of regenerative ability. This view would fit with the observation that many regenerative mechanisms (but not all) recapitulate those observed during development. Of course, the status of remaining cell types selected for in adult animals can in turn affect the processes occurring in embryogenesis (Figure 3).

This simple model only addresses the evolution of regenerative potential with respect to the ability of adult cells to replace damaged or missing tissue. In addition, mechanisms to trigger regeneration, sense what is missing/damaged and ensure pattern and function is properly restored must also evolve. These other parts of the process can also evolve to become less or more efficient, causing changes in regenerative capacity.

Below we cover the major invertebrate model systems and the state of their development as experimental systems, with a focus on highlighting species where robust functional studies are or should now be possible (Alvarado and Tsonis 2006). In doing so we also consider the progress towards answering the question of the origin and identity of the adult stem cells that fuel regeneration, and how they evolve in different animals.

Platyhelminthes: Regeneration in Triclad planarians, *Macrostomum lignano* and beyond

Freshwater planarians are currently the most-studied studied individual invertebrate taxa with respect to regeneration with the use of RNAi combined with an expanding set of experimental and 'omic approaches driving rapid progress. Planarian adult stem cells, called neoblasts, are one of the best-characterized invertebrate adult somatic stem cell systems (Aboobaker 2011; Tanaka and Reddien 2011; Wagner et al. 2011; Gehrke and Srivastava 2016; Zhu and Pearson 2016; Kao et al. 2017; Lai et al. 2017). Planarian regeneration is triggered by wound signals, which induces a change in neoblast behavior to proliferate and differentiate to replace missing tissues through which WBR can be achieved from a tiny proportion of starting adult tissue. PIWI expression neoblasts in planarians are distributed across the parenchyma (Reddien, Oviedo, et al. 2005), they are the only mitotically active cells in planarians, are sensitive to gamma

irradiation (Bardeen and Baetjer 1904; Reddien, Bermange, et al. 2005; Reddien, Oviedo, et al. 2005; Blythe et al. 2010; Önal et al. 2012; Solana et al. 2012), can be isolated by fluorescence-activated cell sorting (FACS) (Hayashi et al. 2006; Romero et al. 2012) and can be identified molecularly by a number of different markers (Newmark and Alvarado 2000; Reddien, Oviedo, et al. 2005; Guo et al. 2006; Eisenhoffer et al. 2008; Wang et al. 2010; Aboobaker 2011; Önal et al. 2012; Solana et al. 2012; Aboukhatwa and Aboobaker 2015). Transplantation experiments performed by injecting single neoblasts into lethally irradiated hosts revealed that at least some individual neoblasts have the ability to repopulate and replace all cell types in an animal (Wagner et al. 2011), and so can be defined as pluripotent. More recently, the development of single-cell transcriptomics has provided strong support for heterogeneity amongst the cycling stem cell population, and it is now widely acknowledged that neoblasts defined as cells positive for *smedwi-1* transcript expression (a *piwi* ortholog) include subclasses (*sigma*, *zeta* and *gamma*) expressing distinct transcription factors (van Wolfswinkel et al. 2014). Amongst these is the *sigma* expressing class that seems to include cells capable of self-renewal, while *Smedwi-1+ zeta* and *gamma* subclasses only pass through M-phase once before terminally differentiating (van Wolfswinkel et al. 2014; Lai et al. 2017). Significantly, a number of regulatory features of planarians stem cells seem to be conserved with mammals, suggesting they are useful model for bio-medically pertinent questions but also that many features of stem cell biology are deeply conserved (Solana et al. 2016; Abnave et al. 2017), including epigenetic regulation (Scimone et al. 2010; Jaber-Hijazi et al. 2013; Duncan et al. 2015; Kao et al. 2017; Mihaylova et al. 2017). While many planarians are capable of WBR, there are some species where regenerative ability is reduced. These scenarios provide an opportunity to understand how differences in regenerative ability evolve, both mechanistically through, for example, changes in key signaling pathways (Liu et al. 2013; Sikes and Newmark 2013; Umesono et al. 2013) and also to understand the evolutionary forces that drive and maintain these differences.

Beyond planarians, another experimental model *Macrostomum lignano* (Macrostomida) has begun to gain momentum. *M. lignano* is a transparent free-living, cross-fertilizing hermaphrodite and is only distantly related to freshwater planarians within the Platyhelminth phylogeny (Schärer and Ladurner 2003; Laumer et al. 2015). *M. lignano* can regenerate all tissue types except its head (Egger et al. 2006; Nimeth et al. 2007) and similarly to planarians this ability is underpinned by neoblasts (Ladurner et al. 2000; Nimeth et al. 2004; Ladurner et al. 2008). Genome and transcriptome sequences of *M. lignano* are now available (Wasik et al. 2015; Grudniewska et al. 2016; Wudarski et al. 2017) and an attractive feature that sets *M. lignano* apart

from planarians is that it has an accessible embryonic life history that has made it amenable to transgenesis (Marie-Orleach et al. 2014; Marie-Orleach et al. 2016; Wudarski et al. 2017), a technology conspicuously still unavailable in planarians. While research so far has focused very much on tool development, *M. lignano* will be particularly powerful for understanding neoblast biology now that these cells and their progeny can be theoretically specifically labeled and followed in live animals.

The discovery that the parasitic platyhelminthes *Schistosoma mansoni* and *Echinococcus multilocularis* also have adult stem cells that look like the neoblasts, makes it very likely that neoblast like-cells are an ancestral character of whole the phylum (Collins III et al. 2013; Brehm and Koziol 2014; Schubert et al. 2014). While *piwi* family genes are the most studied other GMP genes like *vasa, bruno, tudor* and *pumilio* are also expressed in flatworm neoblasts (Shibata et al. 1999; Reddien, Oviedo, et al. 2005; Salvetti et al. 2005; Guo et al. 2006; Rossi et al. 2006; Pfister et al. 2007; Palakodeti et al. 2008; De Mulder et al. 2009; Solana et al. 2009; Shibata et al. 2016). The absence of GMP genes (*Piwi* and *Tudor*) in the neoblasts like cells of *S. mansoni* (Collins III et al. 2013) presumably represents a loss of gene expression and, while the significance of this loss is not understood, it is a demonstration of how pluripotent adult stem cells might evolve. In this case perhaps the need for protection from transposable elements by piRNAs is no longer important. Pioneering work on difficult to access planarian embryos has shown that an early *piwi+* but somewhat molecularly distinct cell population arises early in development and gives rise to neoblasts, after initial germ layer specification, as all organs and tissues start to form during development (Solana et al. 2009; Davies et al. 2017). These data suggest that the pluripotent neoblast population in adults may be functionally distinct from pluripotent embryonic stem cells in planarians (Davies et al. 2017).

Regeneration in annelids involves multipotent cell populations sharing molecular signatures with primordial germ cells

Some members of Annelida are almost as regenerative as planarians, capable of WBR within the context of their segmented body plans, which has been expertly reviewed recently (Özpolat and Bely 2016). In fact the term neoblasts was originally used to describe large undifferentiated cells with a high nuclear to cytoplasmic ratio in the annelid *Lumbriculus* sp. (Randolph 1891). Within Annelida, a number of taxa appear to have lost or never acquired the ability to regenerate

anteriorly, but posterior regeneration has broadly been maintained (Özpolat and Bely 2016). Both Polychaete and Oligochaete annelid species can perform whole-body regeneration as a means of asexual reproduction (Myohara et al. 1999; Bely and Wray 2001; Paulus and Müller 2006; Bely and Nyberg 2010; Bely and Sikes 2010). A wide collection of annelid species can now be cultured in the laboratory. Sexually reproducing species such as *Tubifex, Platynereis, Helobdella, Enchytraeus* and *Capitella* can be taken through their life cycle offering ample embryonic and adult materials while asexual species such as *Pristina, Stylaria, Lumbriculus* and *Enchytraeus* provide genetically identical clonal materials (Özpolat and Bely 2016). The cellular basis of Annelid regeneration is rather varied including examples of differentiated cells switching on GMP gene expression during regeneration in potentially dedifferentiating cells from different germ layers and other examples, like in *Enchytraeus japonensis* where *Piwi* expressing cells are present in the adult migrate to the blastema (Tadokoro et al. 2006; Özpolat and Bely 2016) (Figure 3).

This potential variation in the nature of adult stem cells, in contrast to what is has so far been observed in Platyhelminthes, would make annelids ideal for understanding how the source of adult stem cells during regeneration can change and effect the evolution of regenerative ability (or not). However, functional genomic studies to understand both the cellular source and expression profile of cells driving regeneration are lacking in annelids. Currently *Platynereis*, capable of posterior regeneration involving GMP expressing mesodermal and ectodermal cells, is the most advanced genetic system (Gazave et al. 2013; Bannister et al. 2014; Zantke et al. 2014), but so far the only study of gene function during regeneration has been through RNAi in *E. japonensis* (Takeo et al. 2009; Yoshida-Noro and Tochinai 2010).

Regeneration in the enigmatic worms from Acoelomorpha involves conserved GMP genes

Acoels hold the position of the earliest branching bilaterian taxa (Ruiz-Trillo et al. 1999; Ruiz-Trillo et al. 2002; Telford et al. 2003; Ruiz-Trillo et al. 2004; Philippe et al. 2007; Sempere et al. 2007; Hejnol et al. 2009; Mwinyi et al. 2010). Not unlike platyhelminthes, acoels too possess a neoblast stem cell system that is thought to be collectively totipotent (Sikes and Bely 2010). Several species within the class Acoela have emerged as important models for stem cell and regeneration research and robust molecular genetic tools have started to materialise. Good examples are *Hofstenia miamia* (Srivastava et al. 2014; Gehrke and Srivastava 2016), *Isodiametra pulchra* (De Mulder et al. 2009; Perea-Atienza et al. 2013)*, Symsagittifera roscoffensis* (Bery and Martinez

2011; Bailly et al. 2014; Sprecher et al. 2015) and *Convolutriloba sp.* (Gaerber et al. 2007; Sikes and Bely 2010). *H. miamia* from an early-diverging clade of acoels offers promising avenues for stem cell and regeneration research as RNAi seems effective and, like the flatworm *M. lignano* discussed above, it is possible to access embryos that should facilitate transgenic and genome editing approaches (Srivastava et al. 2014; Gehrke and Srivastava 2016).

Neoblasts in acoels are strikingly similar to those in flatworms, with both systems sharing molecular as well as cellular signatures (Gehrke and Srivastava 2016). As in flatworms, acoel neoblasts have been found to express GMP genes. *I. pulchra* neoblasts express *piwi* and *vasa* (De Mulder et al. 2009) and *piwi* expression was also detected in mitotically active neoblasts in *H. miamia* (Srivastava et al. 2014). Intriguingly, depletion of *piwi* in *H. miamia* (Srivastava et al. 2014) but not in *I. pulchra* (De Mulder et al. 2009) resulted in impaired regeneration.

In addition to similarities in the neoblast population, work with *H. miamia* has uncovered that the pathways responsible for establishing axial polarity during regeneration, such as Wnt signaling, appear to share many features with those uncovered in planarians (Srivastava et al. 2014). Taken together, this supports the hypothesis that an ancestral species at the base of Bilateria may have had both pluripotent stem cells and been capable of axial regeneration using the same conserved signaling pathways. Deeper comparisons between and within Acoels, Platyhelminthes and other taxa are needed to rigorously test this hypothesis.

The study of limb regeneration in arthropods, sources of adult stem cells and somatic piRNAs.

While regeneration amongst insects and crustaceans has been well documented and described, functional work has been limited to just a few species. In *Drosophila*, wound healing and regeneration of imaginal disc tissue, although essentially a developmental tissue has been a very useful model for investigating the response to wounding and the re-establishment of pattern (Bryant and Fraser 1988; Diaz-Benjumea and Cohen 1993; Tabata and Kornberg 1994; Mattila et al. 2004; Bosch et al. 2005). The relatively recent discovery of intestinal stem cells established that *Drosophila* despite being relatively short lived has at least one population of adult stem cells (Micchelli and Perrimon 2006; Ohlstein and Spradling 2006; Ohlstein and Spradling 2007; Lin et al. 2008; Amcheslavsky et al. 2009). Given the tools available in *Drosophila* it is not surprising that the understanding of how these stem cells maintain the barrier function of the midgut has progressed rapidly.

The widely studied two-spotted cricket *Gryllus bimaculatus* has become a relatively mature model for limb regeneration, dependent almost entirely on the work of Noji and coworkers (Mito and Noji 2008). The system permits the effective use of RNAi after limb amputation allowing the effects of knockdown on regeneration to be studied specifically (Nakamura et al. 2008; Bando et al. 2009). This has allowed precise phenotypic analysis of loss of function phenotypes. Coupled with knowledge of gene expression patterns in the regenerating limb this has allowed the role of a number of conserved signaling pathways in limb regeneration to be described (Nakamura et al. 2007; Nakamura et al. 2008; Bando et al. 2009; Bando et al. 2013) and evidence that changes in epigenetic status are also necessary (Hamada et al. 2015). Currently, not much is known about the cellular basis or the nature of cells in the regenerative tissue underpinning limb regeneration in the cricket. The recent demonstration of successful genome editing in the species should help address this deficit (Watanabe et al. 2017).

Studies of the regeneration capacity of crustaceans, including startling observations like new brain neurons forming from circulating hemocytes in crayfish *Procambarus clarkii* and *Pacifastacus leniusculus* (Benton et al. 2014), have remained descriptive in the absence of a genetic system amenable to functional studies. However, the steady establishment of the amphipod crustacean *Parhyale hawaiensis* as a powerful model system for many biological questions (Kao et al. 2016; Martin et al. 2016; Serano et al. 2016; Stamataki and Pavlopoulos 2016), including regeneration, has catered to this shortfall. Studies of limb regeneration have established a description of the tissue and cell level processes that occur during limb regeneration in this species (Konstantinides and Averof 2014). Taking advantage of the lineage specific transgenes facilitated by the germ layer specific cell lineage established by the 8-cell stage of *Parhyale* embryos (Gerberding et al. 2002), it has already been shown that regeneration occurs using cells germ layer specified cells close to the wound. In addition, studies of cells in the limb belonging to the mesodermal lineage revealed the presence of small undifferentiated cells as well as differentiated muscle cells. These cells were found to express a *Pax3/7* transcription factor, a marker of muscle satellite cells that give rise to skeletal muscle and are required for muscle regeneration in vertebrates (Figure 4). Transplantation experiments confirmed that these cells are likely to give rise to new muscle during regeneration. These findings were interpreted as suggesting that ancestor of crustaceans and vertebrates had limbs with *Pax3/7* expressing muscle satellite cells. However, given *Pax3/7* genes labels mesoderm/muscle precursors during development across the Bilateria, it is very possible that vertebrate and arthropod lineages evolved to have *Pax3/7* muscle stem cells in adult limbs independently. A recent study has also

shown the presence of muscle satellite cells in adult *Drosophila* wing flight muscles (Boukhatmi and Bray 2017; Chaturvedi et al. 2017), suggesting they may be conserved across arthropods, adding weight to the argument that the presence of these cells in adults has a deeper evolutionary origin. Overall the striking similarities in limb regeneration suggest detailed study in *Parhyale* will be very informative for understanding vertebrate limb regeneration (Konstantinides and Averof 2014; Alwes et al. 2016; Grillo et al. 2016).

With technological advances such as the availability of fluorescent reporter lines (Pavlopoulos et al. 2009; Kontarakis, Pavlopoulos, et al. 2011; Kontarakis, Konstantinides, et al. 2011; Kontarakis and Pavlopoulos 2014; Wolff et al. 2017), CRISPR-Cas9 based genome editing (Kao et al. 2016; Martin et al. 2016; Serano et al. 2016) and live imaging techniques using transgenic line made using these approaches (Alwes et al. 2016), *P. hawaiensis* has emerged as a very promising model system for addressing regeneration. One limitation has been the relatively large genome size of this species, but this has been partially addressed by a well-annotated draft genome produced by the growing research community (Kao et al. 2016), that will facilitate the use of genome editing.

Available data on the cellular source of regenerative capacity from crayfish *Procambarus* and *Pacifastacus* suggests that circulating hemocytes can contribute to neural regeneration (Benton et al. 2014). In Parhyale, it seems resident germ layer committed precursors fuel limb regeneration. In the case of muscle at least this involves resident stem cells, while other germ layers may require dedifferentiation. Interestingly, even amongst urodelean amphibians this phenotype is variable, as both satellite cells and dedifferentiating muscle fibers re-entering the cell cycle are used in different species for muscle regeneration (Sandoval-Guzman et al. 2014).

There is no direct evidence of the GMP gene expression in somatic cells in arthropods, suggesting this kind of adult stem cell is missing from this phylum and GMP expression is limited to the germline stem cells (GSCs) and the germline. However, this may be because nobody has looked. Recent work has found somatically expressed PIWI-interacting RNAs (piRNAs) across 20 divergent arthropod species, revealing that somatic piRNAs were likely present in the arthropod ancestor (Lewis et al. 2017). Thus it seems more than likely that somatic PIWI expression will also be present. Although *P. hawaiensis* was not amongst those species surveyed it would be an ideal system to confirm the presence of piRNAs in somatic cells (as well as PIWI expression) and study their function in these somatic cells. The possibility that piRNAs and PIWIs are being expressed in somatic adult stem cells in arthropods is exciting and this would add further weight to the argument that these cells are an ancestral feature of metazoan adult life histories.

GMP expressing cells underpin regeneration in deuterostome invertebrates

The phylogenetic placement within Deuterostomia and thus potentially shared genetic machinery with chordates make echinoderms and tunicates attractive invertebrate models for studying regenerative processes from a biomedical perspective. But the sheer variety of potential models and regenerative paradigms also makes them ideal for studying the evolution of regeneration. However, studies have been hampered by complex lifecycles being refractory to laboratory culture and a lack of functional tools. This means that most molecular studies remain descriptive of changes during regeneration, particularly in echinoderms. More mechanistic studies have and should be possible, particularly in *Strongylocentrotus purpuratus, Lytechinus variegatus* (Reinardy et al. 2015) and *Ciona intestinalis* where both excellent genomes and functional genomic tools are available, partly due to their long history as developmental models. Two closely related genera of colonial ascidians, *Botryllus* sp. (Voskoboynik and Weissman 2015) and *Botrylloides* sp. (Rinkevich et al. 2007; Brown et al. 2009) are also very promising systems (Kassmer et al. 2016). Successful RNAi and transcriptomic data were also recently reported in the holothuroid sea cucumber *Holothuria glaberrima* (Mashanov et al. 2014; Mashanov, Zueva, and Garcia-Arrarás 2015), in which pioneering work has developed this species as a standout model amongst this group, that is able eviscerate as a form of autotomy and then regenerate the entire complex gut (Garcia-Arrarás et al. 1998; Garcia-Arrarás and Greenberg 2001; San Miguel-Ruiz and Garcia-Arrarás 2007). This species can also regenerate the central nervous system (Mashanov et al. 2013).

Regeneration in echinoderms is not only used for asexual reproduction, but also serves as a survival mechanism against physical damage, partial predation, and escape from predators. Regeneration is present throughout at the most basal class, Crinoidea (sea lilies) (Carnevali et al. 1997; Candia Carnevali et al. 1998; Thorndyke et al. 2001; Patruno et al. 2003), suggesting that this is an ancestral trait of all extant echinoderms represented by four additional classes: Asteroidea (sea stars), Ophiuroidea (brittle stars), Holothuroidea (sea cucumbers) and Echinoidea (sea urchins). The mechanisms of regeneration can vary between different echinoderm species, where some could undergo bi-directional regeneration (a bisected animal can regenerate two complete animals) while others only regenerate unidirectionally (only one half of a bisected animal can regenerate (Rychel and Swalla, 2009). Bi-directional regeneration via spontaneous fission in adults

allows some ophiuroid, asteroid and holothuroid species to reproduce asexually (Emson 1980; Mazzone et al. 2003; McGovern 2003; Rubilar et al. 2005). Holothuroids also have the striking ability to enviscerate as a form of autotomy, where the viscera is expelled wholesale to defend against predators, subsequently followed by complete gut regeneration (Smith Jr and Greenberg 1973; Byrne 1985; Garcia-Arrarás et al. 1999). On the contrary, amongst all echinoderm classes, sea urchins in the adult form have the most limited ability to regenerate and most could only replace their pedicellariae or spines (Heatfield and Travis 1975; Drager et al. 1989; Dubois and Ameye 2001). This is consistent with the more established model organisms that have proved to be powerful developmental study systems being relatively poor for studying regeneration.

As the closest living relatives of vertebrates (Bourlat et al. 2006), marine ascidians (sea squirts) occupying a basal position in Chordata offer ample opportunities for research on stem cell biology, regeneration and development. Ascidians have two distinct adult body plans, solitary and colonial. Colonial ascidians such as *Botryllus* (Watkins 1958; Laird and Weissman 2004; Voskoboynik et al. 2013), *Perophora* (Goldin 1948; Berrill 1951; Freeman 1964) and *Botrylloides* (Hirose et al. 1995; Rinkevich et al. 2007; Brown et al. 2009) reproduce asexually and therefore can undergo whole-body regeneration (Berrill 1951; Tiozzo et al. 2008; Brown and Swalla 2012). On the contrary, solitary ascidians like *Ciona intestinalis* and those that only reproduce sexually can only regenerate to replace specific body parts, which is somewhat limited compared to colonial species. The transition between colonial and solitary life histories may have evolved multiple times independently since both forms are present across all three ascidian suborders (Phlebobranchia, Aplousobranchia, Stolidobranchia) (Zeng et al. 2006).

Research on *Ciona* regeneration dates back to the early 20th century where historical evidence from earlier studies have demonstrated that Ciona can regenerate its central nervous system, tunic and gonad (Sutton 1953). With the availability of a complete draft genome (Dehal et al. 2002), transgenesis techniques (Awazu et al. 2007; Sasakura 2007; Sasaki et al. 2014; Stolfi et al. 2014) and improvements in laboratory husbandry (Joly et al. 2007), *C. intestinalis* has become one of the most widely studied ascidians. Brain and nervous system regeneration is now one of the primary focuses of research in *Ciona* since complete central nervous system (CNS) regeneration is uncommon in most adult chordates. *Ciona* can regenerate an entire neural complex (NC) over the course of a month (Bollner et al. 1992; Bollner et al. 1997) and with stereomicroscopy analysis and live-imaging techniques, the kinetics of nerve restoration in *Ciona* can now be studied (Dahlberg et al. 2009). Intriguingly, the regenerated NC in *Ciona* is significantly smaller than the original NC, which raises the question as to whether this new cerebral ganglion

has retained its original level of function (Bollner et al. 1992; Bollner et al. 1997; Dahlberg et al. 2009).

The source of stem cells for regeneration includes both circulating and tissue resident stem cells in echinoderms (Thorndyke et al. 2001; Carnevali 2006; Candia-Carnevali et al. 2009). In *H. glaberrima, piwi* transcript is expressed in cells that likely contribute to CNS regeneration and in cells in the neural parenchyma, which may therefore be GMP expressing stem cells (Mashanov, Zueva, and Garcia-Arraras 2015). Data for somatic expression of *Piwi* in other echinoderms is currently lacking. GMP gene expression is well established in somatic stem cells in colonial ascidians. In *Botyrlloides leachii* and *Botryllus Schlosseri* Piwi+ cells are present on the luminal side of the vascular endothelium and endostyle niche respectively (Rinkevich et al. 2010; Rinkevich et al. 2013). In *B. leachii* upon wounding/damage seemingly dormant stem cells in the vascalature, they change morphology, switch on *Piwi* expression, mobilize proliferate and differentiate to allow regeneration (Rinkevich et al. 2010). Knockdown of *Piwi* by siRNAi treatment prevents WBR (Rinkevich et al. 2010). Overall observation of *Piwi*+ cells during regeneration in the sea cucumber and ascidians provides strong evidence they are necessary CNS regeneration, WBR and asexual reproduction by regeneration and confirms that GMP expressing adult stem cells also contribute to regeneration in Deuterostomes.

Stem cell systems in basal metazoans express GMP genes

The phyla Cnidaria and Porifera represent valuable early branching lineages providing key data for understanding the evolution of regenerative ability in metazoans. Cnidaria are divided into five classes; Hydrozoa, Anthozoa, Cubozoa, Staurozoa and Scyphozoa (Figure 5) (Steele et al. 2011), and are well known for their remarkable life history in which they display phenotypic plasticity and extreme regenerative capacity (Holstein et al. 2003; Lengfeld et al. 2009; Steele et al. 2011; Gold and Jacobs 2013; Petersen et al. 2015; Leclère et al. 2016). The main cnidarian model systems, which are *Hydra* (Gierer et al. 1972; Bosch and David 1987; Bosch 2007; Chapman et al. 2010), *Nematostella* (Darling et al. 2005; Reitzel et al. 2007; Burton and Finnerty 2009) and *Hydractinia* (Toth 1967; Duffy et al. 2010; Plickert et al. 2012; Bradshaw et al. 2015; Gahan et al. 2016) have already contributed to our broad understanding of regenerative mechanisms, and comparison between them provides an opportunity to study changes in regeneration at a macro-evolutionary scale over which homology of much adult morphology, tissues and cell types can be assigned.

Regeneration in the hydrozoans, *Hydra* and *Hydractinia*, involves three stem cell systems: endodermal epithelial stem cells, ectodermal epithelial stem cells and interstitial cells (i-cells) (Fig. 2). The two epithelial cell systems are specific for replacing cells from the endodermal and ectodermal lineages (Leclère et al. 2016). I-cells, made up of pluripotent or multipotent cells express both GMP genes (*Piwi, Vasa* and *Nanos*) as well as genes known to be responsible for pluripotency maintenance such as *Polynem* (POU-domain) and *Sox*-family transcription factors (Rebscher et al. 2008; Jager et al. 2011; Millane et al. 2011; Hemmrich et al. 2012; Leclère et al. 2012; Kanska and Frank 2013; Bradshaw et al. 2015) (Figure 5). *Piwi* is expressed in the i-cells of *Hydra* (Juliano et al. 2014; Lim et al. 2014) and *Hydractinia* (Plickert et al. 2012) while *Cniwi* is expressed in the germline of another species from the Hydractiniidae family, *Podocoryne carnea* (Seipel et al. 2003). I-cells in *Hydra* give rise to distinct lineages such as germ cells and neurons (David and Murphy 1977; Sugiyama and Fujisawa 1978; Bosch and David 1987; Siebert et al. 2008; David 2012; Buzgariu et al. 2014) whereas in *Hydractinia*, i-cells collectively regenerate both somatic and germ lineages, including the ectoderm and endoderm (Müller et al. 2004; Duffy et al. 2010; Gahan et al. 2016), so can be considered to be truly pluripotent. Gene expression profiling has revealed that the molecular signatures of i-cells in *Hydra* as defined by morphology, are highly heterogeneous and include sub-populations expressing lineage specific markers (Holstein and David 1986; Hager and David 1997), and a distinct sub-population entirely committed to producing germ cells (Littlefield 1991; Nishimiya-Fujisawa and Sugiyama 1993).

Although the exact nature of adult stem cells is not currently known in *Nematostella*, piRNA pathway components were enriched in both the germline and somatic cells (Praher et al. 2017), implying the existence of putative adult stem cell populations potentially underpinning regeneration in this species. Injury in *Nematostella* activates a quiescent population of mitotic stem cells adjacent to the wound site (Passamaneck and Martindale 2012) and oral regeneration involves the canonical *Wnt* signalling pathway in primary oral-aboral (O-A) axis patterning (Trevino et al. 2011). The inhibition of B-catenin degradation through alsterpaullone treatment results in ectopic regeneration of the oral pole (Trevino et al. 2011). *Wnt* signalling in axial regeneration is also implicated in both *Hydractinia* (Teo et al. 2006; Duffy et al. 2010) and *Hydra* (Hobmayer et al. 2000; Broun et al. 2005; Guder et al. 2006; Lengfeld et al. 2009).

Data from Porifera, the other basal animal lineage, show that *Piwi* is also expressed in the archeocytes of sponges that can give rise to both germ and somatic cells (Funayama et al. 2010).

Piwi is also expressed in mitotically active cells in the tentacles of the hydrozoan *Clytia hemisphaerica* (Denker et al. 2008) and in the ctenophore *Pleurobrachia pileus* (Alié et al. 2011). These data imply that GMP expressing pluri- and multipotent adult stem cells are present in most non-bilaterian taxa, suggesting they are an ancestral feature of metazoan.

Conclusions

The rise of experimentally tractable invertebrate models for regeneration offers unprecedented insights into deciphering fundamental questions in the evolution of genetic and cellular components of regeneration that was not previously possible with most traditional animal models. In order to understand how regeneration evolved there is now an increasing need for understanding environmental and ecological conditions that can lead to the evolution of regenerative ability, and in each case how regeneration is adaptive. It seems likely that a high regenerative ability is an ancestral trait of the Metazoa and this was dependent on the presence of adult stem cells that have many features in common with totipotent GSCs. Molecular comparison of these adult stem cells suggests they are related (Alié et al. 2015), but as they likely all share a GSC origin, this is not necessarily conclusive evidence that they arose once in evolutionary history. Answering this question will require deeper comparisons of mechanisms regulating pluripotency. While this high regenerative ability has been subsequently lost in some taxa is not yet clear whether, once lost, WBR can re-evolve, although many lineages have specific innovations that allow high regenerative capacity of individual organs and tissues (Mount et al. 2006; Li et al. 2007; Kierdorf et al. 2009; Seifert et al. 2012; Szabó and Ferrier 2014).

We propose that one useful way to proceed is to consider the evolution of the adult cell types that remain after development as a target of selective forces that might decide resource allocation between somatic tissues and the germline (Figure 3). While this will not be the only selective force it is likely to be a major influence across most taxa. At one extreme, highly regenerative animals with potentially immortal soma, like *Hydra* and planarians, will maintain pluripotent animals stem cells. Or in their absence we predict that in some animals committed cells will have the potential to naturally access regulatory pathways that allow potency to be restored *de novo*. At the other extreme, animals adapted to have short explosive reproductive cycles may do away with the cost of adult stem cells to remain competitive, with the nematode *C. elegans* perhaps being the best example of this (Figure 3).

In the near future, we can now take advantage of new genomic approaches that do not respect the traditional model organism barriers to understand regeneration and how it evolves. While several technical challenges remain in many for functional studies the importance of the questions merit continued investment.

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Figure legends

Figure 1. Regeneration and stem cells in Metazoa. The ability to regenerate is spread through nearly all animal phyla. Some animals can perform whole body regeneration, while others can only regenerate specialized tissue types, organs or appendages. Some of the current experimental model systems representative of each phylum are depicted. Amongst regenerative animals, pluripotent stem cells or multipotent cells that can give rise to newly regenerated tissue have been identified in some lineages. Expression of the Germline Multipotency Program (GMP) gene *Piwi* in somatic stem cells and/or germline is also noted. The asterisk denotes that *C. elegans* can undergo axon regrowth as a limited form of regeneration without cell proliferation.

Figure 2. Functional genomic tools for invertebrate models of regeneration. The availability of transgenesis techniques: e.g. CRISPR, TALENS, transposon-mediated or I-SceI meganuclease-mediated (in *Nematostella*), RNA interference and high-throughput sequencing datasets (genome and/or transcriptome) are indicated for some current (regeneration) models in each Phylum.

Figure 3. Model depicting the various evolutionary scenario that drive changes in cell lineages in adulthood. (A) During embryogenesis, the totipotent zygote gives rise to intermediate pluripotent and multipotent cell stages during development. Different organisms produce the germline GSCs at different times using different mechanisms, broadly divided into preformation (early and directly descended from the zygote) and epigenesis (induced later by signalling from somatic cells). In adulthood, not only is the persistence of each cell lineage variable, the ability of existing cells to recapitulate genetic regulatory programmes during embryogenesis would also influence the extent of regenerative ability. **(B)** The first hypothetical scenario illustrates extreme cell plasticity where lineages related to stem cell that arise in development are maintained in the adult, and in addition differentiated cells (DC) are capable to de-differentiate back into the multipotent or even the pluripotent state. Currently there are no clear examples known where this could be the case in the Animal Kingdom **(C)** A now well described example of the presence of adult pluripotent stem cells (PSC) are neoblasts in planarians. Here we propose a partially supported model and break the neoblast compartment up by gene expression heterogeneity and capacity for self-renewal into self-renewing *sigma* (σ) neoblasts (PSC) and into *zeta (*ζ), *gamma* (γ) and *nu* (ν) (MPC) which themselves arise from the *sigma* population but then are only able to pass

through mitosis once to give rise to post-mitotic lineage committed progeny (LC), which differentiate into differentiated cells (DC). The perpendicular dotted line after PSCs indicates both that after this point cells are post-mitotic/incapable of self-renewal and that there is no re-entry into the cell cycle. **(D)** The second hypothetical scenario illustrates a situation where DCs are able de-differentiate back into LCs, MPCs or PSCs when regeneration is induced by example for asexual reproduction or injury. However, MPCs and PSCs are only reconstituted to transiently to drive regeneration. **(E)** The third hypothetical scenario illustrates the capability of DCs to de-differentiate back to LCs during regeneration giving extensive regeneration ability (for example limb regeneration) but perhaps not WBR. **(F)** The fourth hypothetical scenario illustrates a situation where DCs could transdifferentiate into another differentiated cell type, with the circular arrows indicating the possibility of each DC lineage able to self-renew. **(G)** The adult nematode possesses only GSCs and DCs. The perpendicular dotted line indicated that apart from GSCs producing gametes, the adult animal is post-mitotic. The expression of the Germline Multipotency Program (GMP) gene which is correlated with stem cell pluri- and multipotency is depicted as a brown bar, with shading indicative of level/extent of expression in a cell population. Dotted arrows denote hypothetical events during a regenerative response. Solid arrows represent cell lineages. Cell lineages that are normally absent in the adult are above the line and in grey when regulatory pathways to reconstitute them cannot be activated in the adult.

Figure 4. Comparison between limb regeneration processes in an arthropod (*Parhyale*) and vertebrate. (A) *Parhyale* limb regeneration involves multiple phases defined by distinct genetic and cellular events; adapted from (Alwes et al. 2016). Following amputation, wound closure ensues through the formation of a melanized scab around the limb epithelium. Muscle tissues located near the distal limb stump subsequently undergo degeneration. Next, a rapid onset of cell proliferation occurs, resulting in morphogenesis and growth of the limb primordium that sits within the limb stump. The final stage of *Parhyale* limb regeneration involves the moulting process, which releases the newly regenerated limb. **(B)** Epimorphic regeneration of the salamander limb (Morrison et al. 2006; Morrison et al. 2010; Sandoval-Guzmán et al. 2014). Following amputation, wound closure and re-epithelialization occurs. Subsequently, cells de-differentiate to form a blastema. Cells within the blastema continue to proliferate, acquire patterning information and differentiate to regenerate a new limb. Muscle regeneration in *Parhyale* and vertebrates involves *Pax7+* satellite cells. In vertebrates, satellite cells reside in a

quiescent state in undamaged muscles where they express *Pax7* and *Myf5*. When muscles are damaged, quiescent satellite cells are reactivated and this is signified by the onset of *MyoD* expression in vertebrates. *Myogenin* (*MyoG*) is expressed when the cells are committed to differentiate to regenerate skeletal muscles (positive for *Mlc3f* expression). In *Parhyale*, *Pax3/7+* satellite cells also represent cellular sources of regenerating muscles. From experiments using *Parhyale* reporter lines, it is thought that *Pax3/7+* is followed by *MyoD* expression upon the onset of muscle formation (Konstantinides and Averof 2014).

Figure 5. Regeneration and stem cells in cnidarians. (A) The phylogeny of Cnidaria showing Anthozoa as an outgroup to Hydrozoa. The subphylum Medusozoa is indicated in a yellow box, which comprises of Hydrozoa, Scyphozoa, Staurozoa and Cubozoa. **(B)** The nature of *Nematostella* stem cells is not currently known. *Nematostella* has unique structures known as mesenteries, which consists of cnidocytes, gonads and myoepithelial cells, allowing the animal to contract along the oral-arboral axis. GMP gene expression has been detected in the mesenteries (Extavour et al. 2005; Praher et al. 2017). Cartoon summarising cell lineages and i-cells in hydroid cnidarians: **(C)** *Hydra* and **(D)** *Hydractinia;* adapted from (Leclère et al. 2016). I-cells, located in interstitial spaces of ephithelial cells, are undifferentiated cells capable of self-renewal, migration and differentiation into specialised cell types. All three cell lineages (ectoderm, ectoderm and i-cells) have self-renewal capabilities. In *Hydractinia* but not in *Hydra*, i-cells can give rise to epithelial cells (ectoderm and endoderm). GMP gene expression is detected in the i-cells of *Hydractinia* (Rebscher et al. 2008; Bradshaw et al. 2015) and *Hydra* (Juliano et al. 2014).

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