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Hydra as a tractable, long-lived model system for senescence

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Hydra represents a unique model system for the study of senescence, with the opportunity for the comparison of non-aging and induced senescence. *Hydra* maintains three stem cell lineages, used for continuous tissue morphogenesis and replacement. Recent work has elucidated the roles of the insulin/IGF-1 signaling target FoxO, of Myc proteins, and of PIWI proteins in *Hydra* stem cells. Under laboratory culture conditions, *Hydra vulgaris* show no signs of aging even under long-term study. In contrast, *Hydra oligactis* can be experimentally induced to undergo reproduction-associated senescence. This provides a powerful comparative system for future studies.

Keywords: *Hydra*; senescence; FoxO; piRNAs; stem cells; longevity

Introduction

Changes in adult somatic stem cell populations are associated with multiple aspects of mammalian age-related physiological decline. Self-renewal and differentiation of at least some somatic stem cells become abnormal with age, and the ability of stem cells to regenerate diverse tissues declines with age in both humans and mice (Sahin & DePinho 2010). Tractable invertebrate models of stem cell maintenance are critical for the understanding of aging. The freshwater cnidarians in the genus *Hydra* are well suited for the study of stem cell maintenance and thus have the potential to play important roles in aging research. Furthermore, *Hydra* possess unique properties including apparent biological immortality and inducible senescence.

Hydra cell and tissue dynamics have long been the subject of study (Bode 1996), with *Hydra* possessing extraordinary regenerative abilities, a property which is likely related to the fact that these animals routinely reproduce asexually (Bode 2003). New experimental techniques and resources have been developed in recent years. The genome of *Hydra magnipapillata* has been sequenced (Chapman et al. 2010). Recent phylogenetic studies indicate that *H. magnipapillata* is closely related to, and very likely the same species as, *Hydra vulgaris* from Europe (Martínez et al. 2010). Stably transgenic *H. vulgaris* can readily be produced by injecting plasmid DNA into the cytoplasm of cells of early embryonic cells (Wittlieb et al. 2006). Transgenic animals expressing fluorescent reporter proteins have substantially facilitated work both on tissue dynamics (Wittlieb et al. 2006) and, through the use of reporter-fusion proteins, on protein localization (Khalturin et al. 2007; Bridge et al. 2010; Fraune et al. 2010; Gee et al. 2010; Nakamura et al. 2011; Boehm et al. 2012; Khan et al. 2013; Lim et al. 2014). Juliano et al. (2014) showed that *Hydra* can be separated into cell lineages via FACS, facilitated by sorting based upon different fluorescent protein transgenes across lineages. Furthermore, powerful reverse genetics approaches can now be implemented, as gene knockdown has been robustly accomplished via transgenic *Hydra* expressing small hairpin RNA to gene targets of interest (Boehm et al. 2012; Juliano et al. 2014).

Morphology and stem cell populations

Radially symmetrical *Hydra* possess a tube-shaped body with the mouth surrounded by tentacles on one end, with the other pole having an adhesive basal disk used to attach to the substrate. The body of a *Hydra* is made up of two epithelial layers, the ectodermal and endodermal epithelia, separated by extracellular matrix. Intercalated between ectodermal cells are interstitial stem cells. Interstitial stem cells give rise to neurons, nematocytes (stinging cells), secretory cells, and gametes (David & Murphy 1977; Bosch & David 1987) but not to epithelial cells. The epithelial and interstitial cells of the body column are mitotically active (Dübel et al. 1987; Holstein et al. 1991). Cell division in the body column displaces epithelial cells toward the ends of the body and into buds (Campbell 1967). Upon reaching extremities, cells become post-mitotic (Dübel et al. 1987; Holstein et al. 1991) The ectodermal and endodermal epithelial cells of the body

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column show structurally specialized features, including muscle fibers adjacent to the extracellular matrix. However, body column epithelial cells also have properties of stem cells. They are self-renewing and can give rise to the different terminally differentiated cell types present in the tentacles, the region adjacent to the mouth, and the foot (reviewed in Hobmayer et al. (2012)).

Longevity

There has been long-standing interest in whether *Hydra* experiences senescence, physiological deterioration with increasing age, leading to reduced reproductive rates and increased mortality rates over time. A study of individual lifespan in *H. vulgaris* provides evidence that members of this species, in fact, are not subject to senescence (Martínez 1998). Mortality rates were determined for three *H. vulgaris* cohorts over a period of four years (Martínez 1998). Animals within these cohorts reproduced both asexually and sexually during the experiment. Age-specific mortality rates remained close to zero throughout the study period. Maximum lifespan in animals is correlated with age at first reproduction (Martínez 1998). Based on the age of first reproduction of *H. vulgaris*, if senescence occurred in this species, a significant increase in age-specific mortality would have been expected over the course of four years (Martínez 1998). These results therefore suggest that *H. vulgaris* may not show senescence. The finding that members of this species have a maximum lifespan of greater than four years in itself demonstrates that *H. vulgaris* can be used to study stem cell populations maintained over relatively long periods of time.

Since *Hydra* reproduce asexually, the interstitial stem cells of an animal give rise to the interstitial stem cells of its asexual progeny and those of their asexual progeny. The same is true for the fate of body column epithelial cells of clonal offspring. This stands in contrast to typical sexual reproductive strategies, with all cell types deriving from a single cell. Recent work by Jones et al. (2014) identifies impressive diversity in aging regimes across animal and plant phyla. These findings stand in contrast to the standard theory of senescence in which mortality increases and fertility decreases with age, as in the disposable soma theory (Kirkwood 1977). *H. vulgaris* are a particularly stark outlier, exhibiting an estimated laboratory lifespan of 1400 years, with constant rates of fertility and mortality throughout (Jones et al. 2014).

The patterns observed in *H. vulgaris* differ substantially from another species of *Hydra*, *Hydra oligactis*, which appears to undergo senescence following the induction of sexual reproduction (Brien 1960; Burnett & Diehl 1964; Noda 1982; Yoshida et al. 2006). When cultured at approximately 18 °C, *H. oligactis* reproduces asexually (Burnett & Diehl 1964), and cultures maintained for years do not show noticeable mortality. When cultured at approximately 10 °C, *H. oligactis* initiate sexual reproduction (Burnett & Diehl 1964). In the study conducted by Yoshida et al. (2006), when the incubation temperature of cultures of male and female *H. oligactis* was lowered to induce sexual reproduction, an increase in mortality rates occurred around 60 days after the temperature change, and almost all animals had died by 150 days after the temperature change. Interestingly, the number of interstitial stem cells per animal drops dramatically by 30 days of incubation in cold conditions and remains low thereafter (Burnett & Diehl 1964; Yoshida et al. 2006). This drop could be explained in terms of increased production by interstitial stem cells of gamete precursors at the expense of production of interstitial stem cells. In any case, the apparent temperature-inducible senescence seen in *H. oligactis* is accompanied by failure to maintain the numbers of interstitial stem cells present in asexual animals (Yoshida et al. 2006).

A powerful model system for aging

The inducibility of aging via culture at 10 °C in *H. oligactis* (Burnett & Diehl 1964; Yoshida et al. 2006), a species exhibiting no senescence at 18 °C, is a powerful research tool. The switchable senescence of *H. oligactis* may indicate a transition from an asexual reproductive strategy, in which the maintenance of the soma is critical, to a disposable soma mode, with investment in gamete production at the expense of aging. This allows for comparison of biologically immortal *H. vulgaris* with *H. oligactis*, including comparative genomic approaches. Further, the experimental induction of aging via temperature manipulation in *H. oligactis* allows for the comparison of senescent and non-aging phenotypes within a single genotype.

Forkhead Box O, stress, aging, and immunosenescence

FoxO proteins mediate a diverse repertoire of responses to cellular stress and influence organismal longevity across phyla. Expression of FoxO increases tolerance to oxidative stress in diverse taxa, in *Drosophila*, *Cenorhabditis elegans*, and mammals (Honda & Honda 1999; Kops et al. 2002; Nemoto & Finkel 2002; Jünger et al. 2003). FoxO has been shown to be involved in enhanced lifespan in *Drosophila* (Jünger et al. 2003) and *C. elegans* (Kenyon 2010). Inbred mouse lines producing low levels of IGF-1, a hormone upstream of FoxO, exhibit enhanced longevity (Yuan et al. 2009). Furthermore, there are human FoxO3A genotypes associated with extended lifespan (Willcox et al. 2008; Flachsbart et al. 2009).

Given the apparent relevance of FoxO in lifespan across diverse animal taxa, the gene is of clear interest in the immortal *Hydra*. The genome of *H. magnipapillata*

was found to contain a single copy of FoxO (Bridge et al. 2010). FoxO is expressed at high levels in interstitial cells and epithelial stem cells (Boehm et al. 2012, Martínez & Bridge 2012). Bridge et al. (2010) suggest insulin/IGF-1 signaling activity may promote cytoplasmic localization of FoxO, as the inhibition of phosphoinositide 3-kinases (PI3 K), an activator lying upstream of Akt and SGK, leads to increased nuclear localization of FoxO. Additionally, inhibition of the JNK pathway results in nuclear localization of FoxO. One hypothesis regarding the role of FoxO in *Hydra* is that, when localized to the nucleus, it functions to protect interstitial cells over the course of time, which are necessary for continued maintenance of the individual, sexual reproduction, and asexual reproduction (Bridge et al. 2010). Boehm et al. (2012) expand upon this initial functional work on *Hydra* FoxO, finding that terminally differentiated cells contain FoxO localized largely to the cytoplasm, with interstitial cells and body column ectodermal cells exhibiting both nuclear and cytoplasmic localization. The overexpression of FoxO is associated with the expression of stem cell genes in terminally differentiated cells, suggesting that excess FoxO results in terminally differentiated cells acquiring stem cell-like properties (Boehm et al. 2012). On the other hand, FoxO knockdown in epithelial cells exhibits a phenotype consistent with aging: a decrease in cell growth and budding rate, accompanied by increases in expression of genes associated with terminal differentiation (Boehm et al. 2012).

Hydra lack an adaptive immune system as found in the jawed vertebrates, but possess an innate immune system (Miller et al. 2007; Augustin et al. 2009; Bosch et al. 2009). The age-dependent deterioration of innate immunity is an important factor in human aging and loss of homeostasis (reviewed by Solana et al. (2012). Boehm et al. (2012) provide evidence that FoxO modulates the expression of three antimicrobial peptides; expression of hydramacin, periculin2b, and arminin is altered by FoxO knockdown.

Schaible and Sussman (2013) hypothesize that the functional diversification of FoxO in other evolutionary lineages detracted from its role in organismal longevity. This model posits that, in aging taxa, FoxO has been coupled to pathways involved in optimizing reproductive fitness early in life, whereas it remains uncoupled in *Hydra*. Experiments examining whether FoxO affects fertility in *Hydra* would address this hypothesis. Because the onset of aging is associated with sexual reproduction in *H. oligactis*, the role of FoxO in this species is particularly relevant to Schaible and Sussman's model.

Hydra myc proteins

Myc family transcription factors play roles in regulating diverse cellular processes, including cell cycle progression, ribosome biosynthesis, protein synthesis, and metabolism. Myc proteins are involved in controlling proliferation and differentiation of stem cells in both mammals and *Drosophila* (Eilers & Eisenman 2008). Four *myc* genes have been identified in the *H. magnipapillata* genome. Two, *myc1* and *myc2*, encode prototypical Myc proteins. Both are expressed in *Hydra* stem cells. *Myc1* is expressed in interstitial stem cells and in dividing somatic cells, specifically precursors of stinging cells and gland cells, derived from interstitial stem cells (Hartl et al. 2010). Knockdown of *myc1* using siRNA causes an increase in interstitial stem cell proliferation, as does treatment with a chemical inhibitor of Myc activity (Ambrosone et al. 2012). *Myc2* is expressed at high levels in interstitial stem cells as well as in dividing gamete precursor cells. It is also expressed at lower levels in body column ectodermal and endodermal epithelial stem cells (Hartl et al. 2010).

Hydra PIWI proteins

In mammals, zebrafish, and *Drosophila,* PIWI proteins are present in the germline and associated with PIWI-interacting RNAs (piRNAs), generally ranging from 24 to 32 nucleotides in length (Thomson & Lin 2009). PIWI proteins are required for fertility in many animals and have a conserved function in transposon repression (Thomson & Lin 2009; Juliano et al. 2011). Another function for PIWI proteins is the maintenance of germ line stem cells in Drosophila (Thomson & Lin 2009; Juliano et al. 2011). In addition, PIWI proteins are expressed outside of the germ line in some somatic stem cells (Li et al. 2009), but functions here are not well explored (Thomson & Lin 2009; Juliano et al. 2011). Two PIWI genes have recently been identified in the *Hydra* genome, *hywi* and *hyli* (Juliano et al. 2014; Lim et al. 2014). *Hywi* and *hyli* are expressed in interstitial and epithelial stem cells in the body column of *Hydra*, and accumulate in the cytoplasm, suggesting a role in post-transcriptional regulation. Furthermore, knockdown of *hywi* results in breakdown of the epithelium, suggesting that *hywi* is necessary for maintenance of the epithelium, with this effect likely due to the epithelial role of *hywi* in post-transcription regulation (Juliano et al. 2014). Additionally, a role in transposon silencing was elucidated, though this function of Hywi- and Hyli-bound piRNAs appears to be principally present in the interstitial cells (Juliano et al. 2014).

Krishna et al. (2013) investigated the role of small non-coding RNAs in the context of *Hydra* head regeneration, finding that piRNAs are the most common small RNAs in *Hydra*. Significantly, Krishna et al. (2013) found that piRNAs mapping to transposable elements and the transcriptome display a ping-pong signature. This ping-pong signature of amplification represents a

conserved mechanism that was present before the divergence of metazoans (Grimson et al. 2008), in which interactions between complementary regions of sense and antisense piRNAs lead to the amplification of piRNAs, subsequently facilitating cleavage of transposon mRNAs (Brennecke et al. 2007). This represents a conserved mechanism of transposon silencing that was present before the divergence of metazoans (2008). Work by Lim et al. (2014) and Juliano et al. (2014) both support the localization of *Hydra* PIWI proteins to a structure similar to the nuage, an organelle in the vicinity of the nucleus of germline cells in which ping-pong amplification of piRNAs and subsequent transposon silencing take place (Brennecke et al. 2007; Li et al. 2009, 2014). These findings provide further support for a conserved role of piRNA pathway.

It has been proposed that the accrual of transposable elements contributes to aging (Gaubatz & Flores 1990; Murray 1990), reviewed by (Teplyuk 2012). A recent study of transcriptional availability as indicated by chromatin state in human diploid fibroblast cells provides evidence of an association between transposable elements and aging (De Cecco et al. 2013). De Cecco et al. (2013) found that senescent cells have open chromatin associated with transposable elements, leading to higher transcription of transposons and subsequent transposon mobilization. Expressed preferentially in germline cells (Aravin et al. 2006; Gan et al. 2011), piRNAs have established roles in the silencing of transposable elements. Notably, transposable elements constitute approximately 57% of the *H. magnipapillata* genome, with the presence of active transposons demonstrated by their representation in EST sequences (Chapman et al. 2010). Even though this is not an unusual abundance of transposons, with transposable elements representing about half of the human genome and 85% of maize (Lander et al. 2001; Schaible & Sussman 2013), transposons could have a substantial effect on the genome if not kept in check. Indeed, in *Drosophila*, mutants with dysfunctional *Argonaute 2*, a protein which mediates guide RNAs silencing transposons, have shortened lifespan and accelerated age-dependent neuronal decline (Li et al. 2013). Thus, evidence from *Hydra* and other organisms suggests that PIWI proteins may be important in long-term maintenance of *Hydra* stem cells. A curious aspect of the PIWI-piRNA pathway in *Hydra* is that while it appears to target transposons in interstitial stem cells, its targets for post-transcriptional repression in epithelial stem cells appear to be primarily non-transposon genes (Juliano et al. 2014). This is interesting, given the consideration that epithelial stem cells also presumably need to be maintained through the life of a *Hydra* and the lives of all its asexually produced progeny.

Conclusions

Hydra represent a unique model system for the study of senescence, with the opportunity for the comparison of non-aging and induced senescence. This genus is developmentally well characterized, amenable to genetic manipulation, and readily culturable as clonal lines. Recent work has illuminated the roles in *Hydra* stem cells of proteins important in stem cell maintenance in other organisms. Further study of *Hydra* holds potential for the basic science of the evolution of aging and stem cell maintenance, along with potential for translational therapies applicable to human health.

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