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Insulin/IGF-1 Signaling Receptor HTK7 Expression in Aging and Non-Aging *Hydra oligactis*

By

Claire C. Weckerly

This thesis is submitted in fulfillment of the requirements for Honors in the Discipline in Biology (Note: Only if completing HID) and the Elizabethtown College Honors Program

May 1, 2019

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Elizabethtown College Department of Biology

Insulin/IGF-1 Signaling Receptor HTK7 Expression in Aging and Non-aging *Hydra oligactis*

Claire Weckerly
5-9-2019

Abstract

The freshwater cnidarians of the genus *Hydra* are a potential system for the study of aging. *Hydra vulgaris* shows negligible senescence. *Hydra oligactis*, on the other hand, shows rapid aging after it has been induced to sexually reproduce. Understanding the onset of aging in *H. oligactis* may provide insights relevant to aging and the accompanying diseases in humans.

One attractive pathway for study is the insulin/IGF-1 signaling (ISS) pathway, a conserved pathway which affects lifespan in both invertebrate and vertebrate model organisms. In *H. vulgaris*, activation of the ISS pathway is thought to reduce activities of the transcription factor FoxO and induce differentiation of stem cells.

The purpose of this experiment was to determine whether expression of the insulin-like peptide receptor HTK7 gene changes in *H. oligactis* once aging is induced. It was expected that HTK7 would be expressed in stem cells in aging *H. oligactis,* causing the pre-mature differentiation of these cells, and contributing to the onset of aging.

Expression of HTK7 was analyzed using whole-mount RNA *in situ* hybridization. *H. oligactis* showed highest HTK7 expression in epithelial cells in regions where differentiation takes place: at the base of the tentacles and adjacent to the basal disk. When analyzing the foot region of the polyps, it was seen that the foot became larger in aging *H. oligactis*. This phenotype mimicked ones previously seen in FoxO-deficient *H. vulgaris*, indicating that HTK7 could be pre-maturely downregulating FoxO in the foot region and contributing to the onset of aging.

Introduction

Hydra is a freshwater cnidarian polyp that is used as a model organism in aging studies. Different species of *Hydra* differ in whether they show senescence or not, which makes them useful for comparative studies of the aging process. *Hydra vulgaris* shows negligible senescence. However, aging can be induced in *Hydra oligactis* by stimulating gametogenesis (Yoshida et al., 2006). Previous experiments have shown that when *H. oligactis* are induced to sexually reproduce by exposure to cold temperatures, they die within 150 days (Yoshida et al., 2006). Physiologically, aging within *H. oligactis* is characterized by a change in morphology and a decline is processes such as prey capture, spontaneous contractions, and gastric functions (Yoshida et al., 2006; Tomczyk et al., 2015).

The regenerative properties of *H. vulgaris* are attributed to its three cell lineages: the ectodermal epithelial cells, the endodermal epithelial cells, and the interstitial cells, which all show the characteristics of stem cells (Hobmayer et al., 2012). These cells divide in the gastric region of the polyp, where they displace existing cells at the ends of the body, either toward the tentacles and the head or toward the basal disk. Once the stem cells reach these outer locations, terminal differentiation occurs, as indicated by changes in morphology and gene expression (Bode, 1996). Despite the dynamic structure of *H. vulgaris*, the body maintains constant spatial organization and proportions of each cell lineage by ensuring that the rate of terminal differentiation and rate of stem cell death are equal (Hobmayer et al., 2012; Bode, 1996).

Insulin/insulin-like growth factor signaling (ISS) has a role in the aging process that is highly conserved, in both invertebrates and vertebrates, including humans (Flatt & Partridge, 2018). Mutational studies that cause loss of function within the ISS pathway have shown that reduced ISS activity is linked to a longer lifespan and resistance to age-related diseases in *Drosophila*, *C. elegans*, and mice (Flatt & Partridge, 2018). Thus, characterizing changes within this pathway that occur during the onset of aging in *H. oligactis* can lead to a better understanding of how to prevent the onset and effects of aging within humans.

In *Drosophila, C. elegans*, and mice, the ISS pathway is initiated when insulin or insulin-like growth factors (IGF-1) are present. These factors bind receptors that will then activate PI3K/Akt/SGK, resulting in the phosphorylation of the transcription factor FoxO (Schaible &

Sussman, 2013). This phosphorylation leads to the cytoplasmic localization and destruction of FoxO, which prevents it from being able to affect transcription (Fanni et al., 2018).

Normally, FoxO upregulates genes that will protect cells from environmental stress such as oxidative stress, thermal stress, or bacterial infections (Flatt & Partridge, 2018). Thus, inhibition of FoxO through ISS activity is thought to be linked to cellular susceptibility to stressors, which may reduce longevity and lead to the onset of senescence (Flatt & Partridge, 2018).

Studies using *H. vulgaris* have suggested that ISS regulation of FoxO is conserved within *Hydra.* The predicted *H. magnipapillata* FoxO sequence has phosphorylation sites that are predicted to be utilized by Akt/SGK (Bridge et al., 2010). The role of ISS in FoxO inhibition has been evidenced by experiments using a PI3K inhibitor. When ISS activity reduced by PI3K inhibition, nuclear localization of FoxO is increased (Bridge et al., 2010). Finally, expression of *Hydra* insulin-like peptide genes has been shown to decrease rates of FoxO-induced apoptosis of epithelial cells (Lasi, David, & Böttger, 2009).

The effects of FoxO reduction in *H. vulgaris*, have been studied using shRNA. Knockdown of FoxO caused decreased stem cell proliferation, terminal differentiation of somatic stem cells, and a slower rate of budding (Boehm et al., 2012). However, decreased FoxO expression did not affect the mortality of *H. vulgaris*, which suggests that other factors may contribute to the lack of senescence observed in this species (Tomczyk et. al 2015).

In order for ISS to start, the presence of insulin/IGF-1 must be detected by receptors. Like *C. elegans* and *Drosophila*, *H. vulgaris* possesses one gene related to both insulin and insulin-like growth factor receptors of vertebrates (Steele et al., 1996). The *H. vulgaris* gene, HTK7, encodes a 4.8 kb transcript (Steele et al., 1996).

The HTK7 receptor shares a number of features with the mammalian insulin receptor and the mammalian IGF-1 receptor, including an extracellular region with conserved cysteine residues, which serves as the ligand-binding site. It also has a conserved catalytic domain, fibronectin type III repeat, and juxtamembrane sequence of NPEY, indicating that the *Hydra* HTK7 receptor is functionally similar to the mammalian insulin and insulin-like growth factor receptors (Steele et al., 1996).

Localization of the HTK7 receptor mRNA within *H. vulgaris* has been analyzed using RT-PCR, *in situ* hybridization, and cell-lineage-specific transcriptome data (Steele et al., 1996;

Hemmrich et al., 2012). *In situ* hybridization indicates that HTK7 receptor mRNA is present in the ectoderm (Steele et al., 1996). Accordingly, cell-lineage-specific transcriptome results imply that HTK7 receptor mRNA is present at lower levels in cells of the endoderm and interstitial cell lineages than in cells of the ectodermal lineage (Hemmrich et al., 2012). The highest HTK7 receptor mRNA expression is seen at the base of the tentacles and adjacent to the basal disk, where cells stop dividing and differentiate (Steele et al., 1996; Bode, 1996). HTK7 is expressed in these same regions in developing buds (Steele et al., 1996).

Though the highest levels of HTK7 mRNA are seen in the newly differentiating cells of *H. vulgaris,* FoxO expression is seen in the undifferentiated cells of the body column (Bridge et al., 2010; Boehm et al., 2012). FoxO-knockdown experiments have shown that a reduction in FoxO induces terminal differentiation. Thus, it is predicted that stem cells will upregulate the HTK7 receptor and downregulate FoxO once they reach the border of differentiation. This is consistent with a role for ISS in the induction of stem cell differentiation. When cells are fully differentiated though, HTK7 signaling seems to be unneeded and the receptor is downregulated again.

While HTK7 and FoxO have been studied in the non-aging species *H. vulgaris*, currently there is no information about the ISS pathway in *H. oligactis*. The purpose of this experiment is to use RNA *in situ* hybridization to determine the expression of the HTK7 receptor in *H. oligactis* before and after the induction of aging. In *H. vulgaris,* reduction in FoxO activity leads to stem cell differentiation. The highest expression of *H. vulgaris* HTK7 receptor mRNA expression is seen at the base of the tentacles and adjacent to the basal disk, where little FoxO expression is seen and terminal differentiation begins to occur.

Thus, it is expected that in aging *H. oligactis,* more expression of HTK7 may be seen in the body column of the polyp. If HTK7 is expressed in the body column, the activities of the HTK7 receptor and ISS may be causing decreases in FoxO activity and pre-maturely inducing differentiation of stem cells, disrupting the dynamic equilibrium of the *H. oligactis* body column, and leading to rapid aging.

Overall, this work will lead to a deeper understanding of *Hydra* as a model for aging. Both the ISS pathway itself and its role in aging are highly conserved (Flatt & Partridge, 2018). As a structurally simple, experimentally tractable model, *Hydra* may provide insight into targets for treatment of diseases of aging in humans.

Materials and Methods

Production of the HTK7 probe

In order to produce a labeled probe complementary to *H. oligactis* HTK7 mRNA, a portion of the *H. oligactis* HTK7 gene was isolated and cloned for use as a template for labeled RNA synthesis. While high-quality genome assemblies for *H. vulgaris* and the marine cnidarian species *Hydractinia symbiolongicarpus* are publicly accessible, this is not true for *H. oligactis*. Therefore, to identify conserved regions of HTK7 for use in designing PCR primers, sequences of the *H. vulgaris* and *Hydractinia* HTK7 genes were compared. It was assumed that these highly conserved regions would also be present in the *H. oligactis* HTK7 gene. The following primers were created: GCGT**C**GTGTTGTGGGAAATATGC (forward, T_m= 59˚) and TACACGGGAATCGGTTTGC (reverse, T_m= 55.4˚). The nucleotide shown underlined and in bold differed between the *H. vulgaris* and the *Hydractinia symbiolongicarpus* gene sequences, so the base present in the *H. vulgaris* sequence was chosen for the primer. PCR reactions with these primers used genomic DNA from the *H. oligactis* Swiss male laboratory strain as a template. The temperatures and times used for PCR were 95˚ C for 2 minutes, followed by four cycles of 94˚ C for 30 seconds, 55˚ C for 1 minute, and 68˚ C for 2 minutes, followed by 31 cycles of 94˚ C for 30 seconds, 50˚ for 1 minute, and 68˚ C for 2 minutes, and ending with 68˚ C for 10 minutes. Gel electrophoresis of the reaction product confirmed the expected size of 676 base pairs.

The PCR product was purified using gel electrophoresis on a 1% TAE gel. The resulting band was extracted using the Qiaquick Gel Extraction Kit (Qiagen). The DNA was ligated into a plasmid using the pGEM-T Easy vector system (Promega). One-Shot Chemically Competent *E. coli* (Invitrogen) were transformed with DNA from the ligation reaction. Plasmid DNA was isolated from three mL overnight Luria broth cultures inoculated from single bacterial colonies, using the QIAprep Spin Miniprep Kit (Qiagen). Plasmid DNA isolation included the recommended wash with Buffer PB. Presence of an insert in the plasmid DNA was determined by digestion with EcoRI followed by gel electrophoresis.

Plasmid DNA from one bacterial colony was selected for probe production. The sequence of the insert in this plasmid was determined by Eurofins MWG. The sequence obtained was used in a BLAST search to determine the direction of the insert within the plasmid. The cloned *H.*

oligactis DNA only showed 87% identity with the *H. vulgaris* DNA sequence. This indicated that DNA from *H. vulgaris* was not amplified and cloned unintentionally .

In order to prepare the DNA for use as a template for RNA probe synthesis, it was linearized using Nco I-HF so that *in vitro* RNA synthesis would stop at the end of the insert DNA and not use plasmid DNA as a template. The cut DNA template was rendered RNase-free through phenol-chloroform extraction. DNA was precipitated using pH 5.2 sodium acetate and ethanol. The pellet obtained was washed with 80% ethanol and resuspended in 10 μL RNase-free water.

The digoxygenin-labeled RNA probe was produced using the HTK7 template DNA and the Roche DIG RNA Labeling Kit, with SP6 polymerase used for transcription. RNA was precipitated using LiCl and ethanol. The pellet obtained was washed with 80% ethanol and resuspended in 20 μL RNase-free water. The RNA was heated to 65° C to resuspend the pellet. To verify the production and integrity of the RNA, 1.5 μL of the RNA was run on an RNase-free 1% agarose gel at 80 volts, after heating to 65° C for five minutes to denature the RNA. A band of the expected size was present, with no smearing below the band, implying that little or no RNA degradation had occurred.

Control probes

Labeled RNA corresponding to a portion of the gene encoding the transcription factor Hyzic was used for controls during the *in situ* hybridization procedures. Hyzic is expressed in differentiating stinging cells of *H. vulgaris* within the body column (Lindgens, Holstein & Technau, 2003). An antisense probe complementary to Hyzic RNA was used as a positive control. A sense probe, produced by transcribing RNA from the same template in the opposite direction, was used as a negative control. Because the sense probe shouldn't bind to any target, it is able to show background staining caused by the *in situ* hybridization procedure.

Hyzic probes were created by Dr. Diane Bridge (Elizabethtown College) using *H. magnipapillata* cDNA. The following primers were used:
GAGAGAAACGCGCTGATTTAACGTAGC (forward, T_m = 59.7˚ C) and
CATGATGCTCGATCCACATACAGGAC (reverse, T_m = 59˚ C). PCR reactions with these primers used genomic DNA from the *H. oligactis* Swiss male laboratory strain as a template. The temperatures and times used for PCR were 95˚ C for 1 minute, followed by ten cycles of 94˚ C

for 30 seconds, a 1 minute annealing step at a temperature that began 58˚ C and dropped by 0.5˚ C each cycle, and 68˚ C for 2 minutes, followed by 30 cycles of 94˚ C for 30 seconds, 53˚ for 1 minute, and 68˚ C for 2 minutes, and ending with 68˚ C for 10 mins. The PCR product was then isolated and cloned using the procedures described for the HTK7 template. DNA was linearized with Sal1 to create the sense probe and with Nco1 to create the antisense probe. RNA was produced from these templates using the procedures described for the HTK7 probe. Both control probes were created on 12/15/10.

Hydra culture conditions and strains

All *Hydra* used in this experiment were cultured at Elizabethtown College. The strains of *Hydra* were used as follows: *Hydra oligactis* (Swiss strain), a male strain of genetically identical hydra, and *Hydra magnipapillata* (strain 105 of *Hydra vulgaris*). *H. oligactis* and *H. magnipapillata* were kept at 18˚ C in a solution of 1 mM $CaCl_2$, 1.5 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.08 mM $MgSO_4$, and 0.03 mM KNO_3. All animals were fed three times a week with freshly hatched brine shrimp. To induce aging within *H. oligactis*, the polyps were moved to 10˚ C. All other culture and feeding practices remained the same. *H. oligactis* incubated at 10˚ C for 27 days and for 42 days, in separate containers, were used in this study.

RNA _in situ_ hybridization

The RNA *in situ* hybridization procedure was repeated in replicate using the treatment groups outlined in Table 1. The procedure outlined in Grens et al. (1996) and Martinez et al. (1997) was followed. The positive control Hyzic antisense probe was used with *H. magnipapillata*, because the probe is complementary to *H. magnipapillata* mRNA. The negative control Hyzic sense probe was not expected to bind to mRNA in either *H. magnipapillata* or *H. oligactis*. It was used with *H. oligactis* in order to detect any non-specific staining produced in *H. oligactis* by the procedure followed.

Imaging

Stained *Hydra* were viewed using a Nikon Eclipse 80i light microscope. Pictures were taken using a Nikon Digital Sight DS-Ri1 camera and analyzed using NIS Elements Imaging Software.

Table 1. Treatment groups for RNA *in situ* hybridization. *H. oligactis* were incubated either at 18˚ C or at 10˚ C for the time indicated. These *H. oligactis* were then used in the HTK7 experimental group and the negative control using the Hyzic sense probe. *H. magnipapillata* were used as the positive control for interstitial cell staining using the Hyzic antisense probe. The hybridization procedure was completed in replicate over two different dates.

Animals Used (Number of Animals)	Temperature treatment of animals (days at 10˚ C)	Probe used (dilution)
Male *H. oligactis* (12)	18˚ C	HTK7 (1:100)
Male *H. oligactis* (24)	18˚ C	HTK7 (1:100)
Male *H. oligactis* (12)	10˚ C (27 days)	HTK7 (1:100)
Male *H. oligactis* (12)	10˚ C (42 days)	HTK7 (1:100)
Male *H. oligactis* (12)	18˚ C	Hyzic sense (1:50)
Male *H. oligactis* (12)	18˚ C	Hyzic sense (1:50)
Male *H. oligactis* (12)	10˚ C (27 days)	Hyzic sense (1:50)
Male *H. oligactis* (12)	10˚ C (42 days)	Hyzic sense (1:50)
Male *H. magnipapillata* (12)	18˚ C	Hyzic antisense (1:50)
Male *H. magnipapillata* (12)	18˚ C	Hyzic antisense (1:50)

Results

Patterns of HTK7 expression in *H. oligactis* incubated at 18˚ C (non-aging) and at 10˚ C for 27 and for 42 days (aging) were qualitatively similar (Figures 1-3). The highest levels of HTK7 expression were at the bases of the tentacles and adjacent to the basal disk. Low levels of expression within the body column were present. It should be noted that there was staining in the negative control animals incubated for 42 days at 10˚ C. However, the staining was present only in cells underlying the testes, while the experimental animals showed staining in the body column cells (Figure 2D).

As seen in Figure 2A and Figure 2C, HTK7 was expressed in the ectoderm cells, because staining was seen on the outer edge of the mesoglea, a layer of extracellular matrix that separates the endoderm from the ectoderm (Hobmayer et al., 2012). HTK7 expression was not detected in cells of the interstitial lineage. Gene expression in interstitial stem cells and differentiating derivatives shows a spotted pattern, with small patches showing high expression, as seen in the positive control (Figure 4).

The only stained animals with asexual buds were in the group incubated at 10˚ C for 27 days (Figure 5). The highest HTK7 expression is seen at the base of the forming tentacles and adjacent to the developing basal disk. Lower expression was seen in the body column. Expression was detected in ectodermal cells of the buds.

An unexpected observation was that the basal disk region below the zone of high HTK7 expression was consistently larger in *H. oligactis* incubated at 10˚ C than animals maintained at 18˚ C (Figure 6). Basal disk cells have a distinctive morphology. They are taller than body column epithelial cells and differ in color because of the presence of secretory vesicles (Davis, 1973) The region of the body occupied by basal disk cells was larger in *H. oligactis* incubated at 10˚ C for 27 and for 42 days than in *H. oligactis* maintained at 18˚ C (Figure 6). However, the border of HTK7 expression remained adjacent to the basal disk tissue in *H. oligactis* incubated at 10˚ C. Thus, the basal disk region of the body without HTK7 staining was correspondingly larger in *H. oligactis* incubated at 10˚ C than in *H. oligactis* maintained at 18˚ C (Figure 6).

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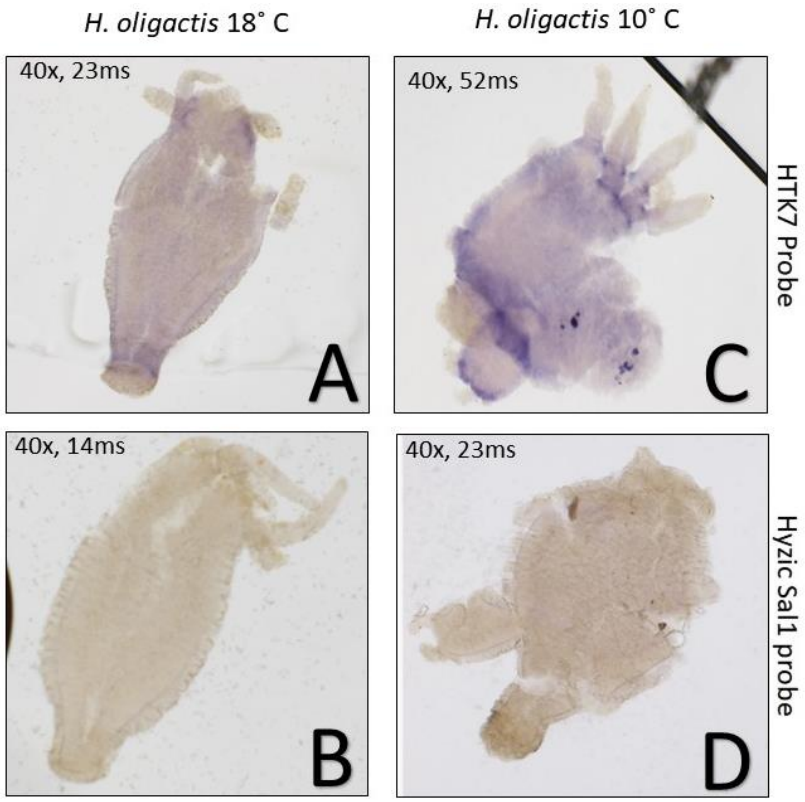

Figure 1. HTK7 expression in *H. oligactis* maintained at 18˚ C and *H. oligactis* incubated at 10˚ C for 42 days. Highest HTK7 expression is seen at the bases of the tentacles and adjacent to the basal disk. Low expression is also seen in the body column. The Hyzic Sal1 probe served as a negative control. It is identical to a portion of the Hyzic mRNA, rather than complementary to the mRNA, and therefore is not expected to bind to any target. Magnification and exposure times are indicated for each image.

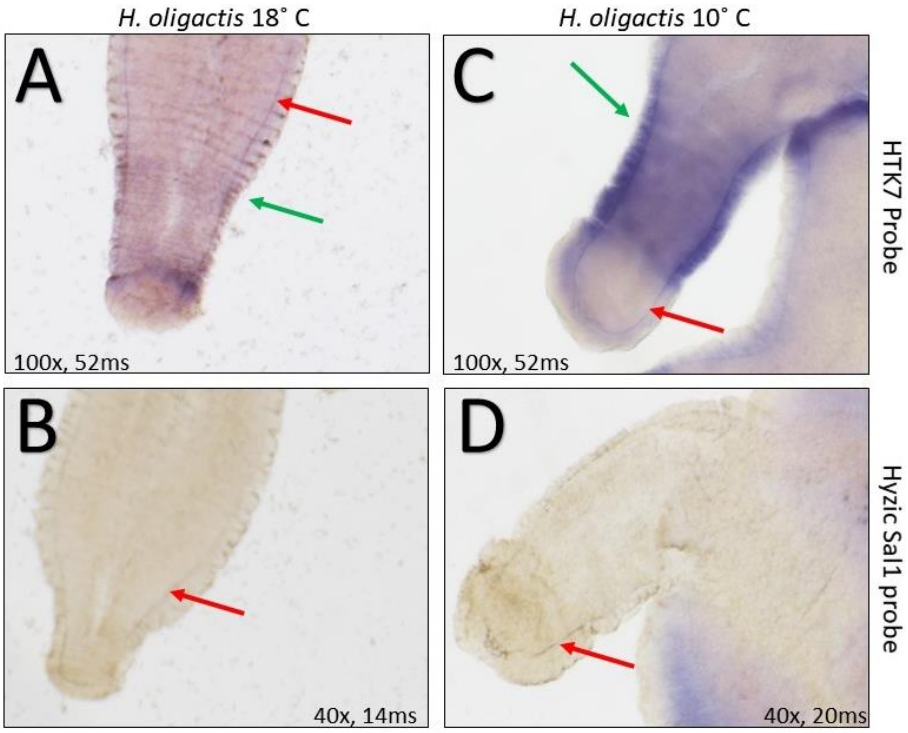

Figure 2. Basal region HTK7 expression in *H. oligactis* maintained at 18˚ C and *H. oligactis* incubated at 10˚ C for 27 days. High expression of HTK7 is seen adjacent to the basal disk, beginning at the point indicated by the green arrow. The red arrow indicates the mesoglea; tissue distal to the mesoglea is ectoderm. The Hyzic Sal1 probe served as a negative control. Magnification and exposure times are indicated for each image.

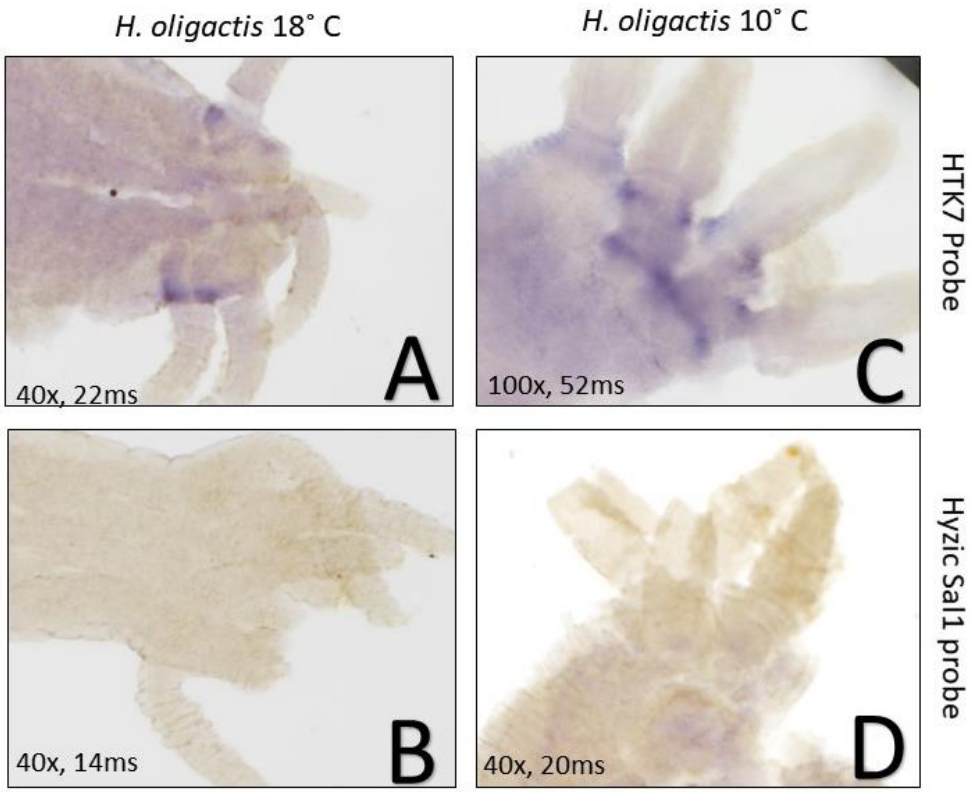

Figure 3. Head region HTK7 expression in *H. oligactis* maintained at 18˚ C and *H. oligactis* incubated at 10˚ C for 42 days. High expression of HTK7 is seen at the base of the tentacles. The Hyzic Sal1 probe served as a negative control. Magnification and exposure times are indicated for each image.

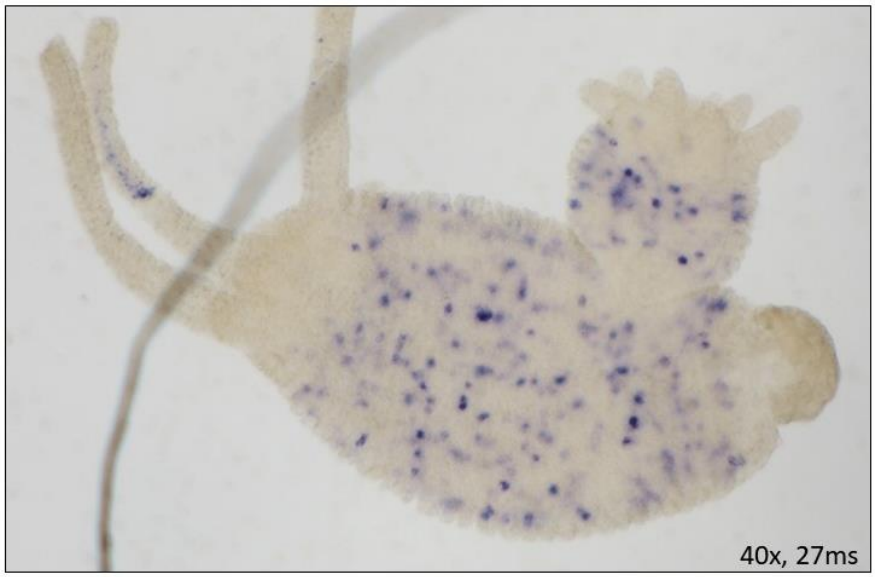

Figure 4. Positive control staining for Hyzic in *H. magnipapillata*. The *H. magnipapillata* Hyzic Nco1 antisense probe was used to detect expression of the transcription factor Hyzic in differentiating stinging cells of the interstitial lineage. Magnification and exposure time are indicated in the lower right corner.

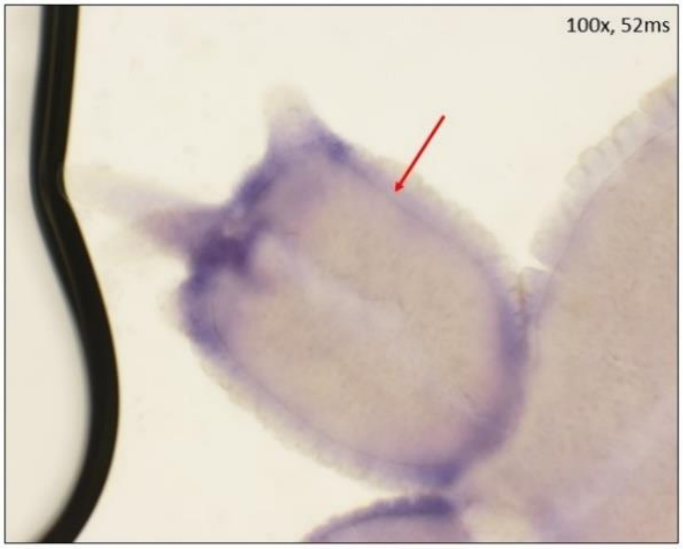

Figure 5. HTK7 expression in a bud of *H. oligactis* incubated at 10˚ C for 27 days. High expression of HTK7 is seen at the base of the tentacles and adjacent to the developing basal disk. The red arrow indicates the mesoglea; tissue distal to the mesoglea is ectoderm. Magnification and exposure time are indicated in the upper right corner.

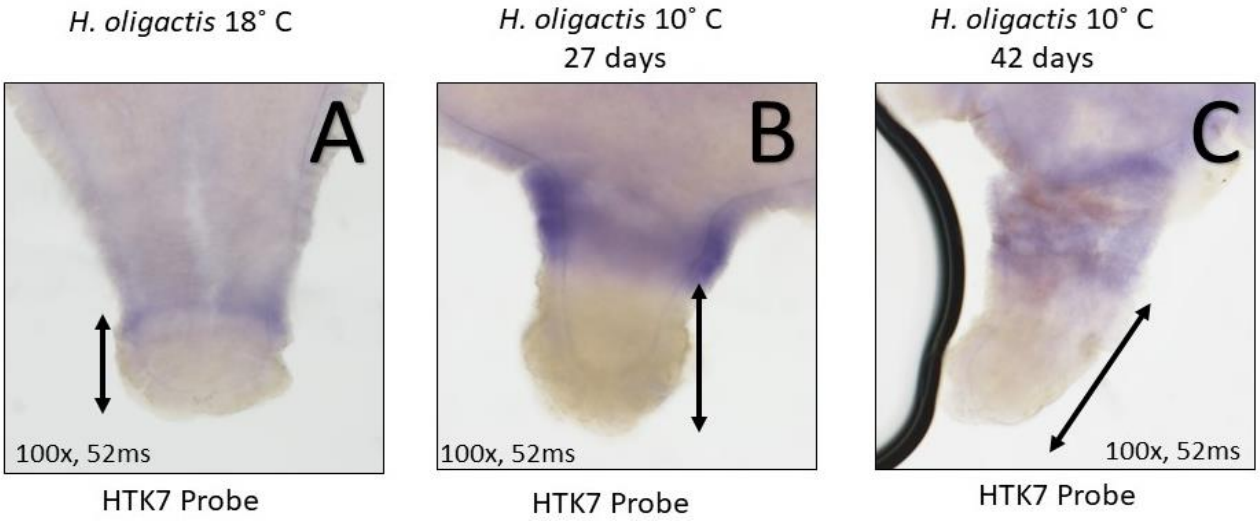

Figure 6. *H. oligactis* foot morphology at different temperature treatments. *H. oligactis* were kept at 18˚ C (nonaging) or incubated at 10˚ C for either 27 days or 42 days (aging). The basal disk appeared larger in animals incubated at 10˚ C. HTK7 expression is high adjacent to the basal disk, so that the basal region without expression is larger in animals incubated at 10˚ C. The black lines indicate the distance between the ring of high HTK7 expression and the aboral end of the body. Magnification and exposure times are indicated for each image.

Discussion

Identical patterns of HTK7 expression were seen in the *H. oligactis* incubated at 10˚ C for 27 days, those incubated at 10˚ C for 42 days, and buds found on the polyps incubated at 10˚ C for 27 days. This pattern of HTK7 expression in non-aging *H. oligactis* was consistent with the pattern seen in aging *H. oligactis* that were maintained at 18˚ C. Highest HTK7 expression was seen at the base of the tentacles and adjacent to the basal disk. HTK7 was expressed in the ectoderm of the *H. oligactis* adults and buds. Because the ectoderm overlays the endoderm and the interstitial cells, no conclusions can be confidently drawn about HTK7 expression in these stem cell lineages.

These results correlate with the expression of HTK7 within *H. vulgaris,* as demonstrated by Steele et al. (1996), who showed that in *H. vulgaris*, high HTK7 expression in occurs in regions where the ectodermal epithelial stem cells of the body column are starting to differentiate. High HTK7 expression at the base of the tentacles and near the basal disk may potentially lead to reduction of FoxO activity, promoting the differentiation of these cells (Boehm et al., 2012). The results also agree with cell-lineage specific transcriptome analyses, which indicate highest expression of HTK7 in the ectoderm of *H. vulgaris* (Hemmrich et al., 2012). Given that the body organizations of *H. vulgaris* and *H. oligactis* are similar, it is not surprising that HTK7 expression is similar in *H. vulgaris* and non-aging *H. oligactis*.

Because the ISS pathway plays a conserved role in aging and stem cell differentiation, it seemed possible that HTK7 expression might change with the onset of aging in *H. oligactis*. One possibility was that aging *H. oligactis* would show increased HTK7 expression in the stem cells of the body column, and this expression would be associated with differentiation and loss of stem cells. However, expression in the body column did not increase to the same level of expression seen in regions of terminal differentiation in the *H. oligactis* incubated at 10˚ C for either 27 or 42 days.

An unexpected result, though, was that in *H. oligactis* incubated at 10˚ C, the basal disk was consistently larger than in *H. oligactis* maintained at 18˚ C. The ectoderm of the basal disk consists of mucous-secreting cells present at the aboral end of *Hydra* (Davis, 1973). In both *H. vulgaris* and *H. oligactis*, HTK7 is expressed in ectoderm cells adjacent to the basal disk. However, the aging *H. oligactis* showed a larger area of cells beneath the region of high HTK7

expression (Figure 6). Furthermore, this area appeared larger in *H. oligactis* that were incubated at 10˚ C for 42 days than in *H. oligactis* incubated at 10˚ C for only 27 days. The feet of *Hydra* are made up of fully differentiated cells (Hobmayer et al., 2012). Hence, the results of this experiment suggest that induction of aging within *H. oligactis* is associated with more stem cell differentiation in the basal disk region, which can be seen as a larger basal disk.

Similar enlargement of the basal disk was observed when FoxO expression was knocked down in *H. vulgaris.* In experiments using shRNA to reduce FoxO expression in epithelial cells, the basal disk was significantly enlarged, and the expression of genes associated with stem cells was reduced near the basal disk region (Boehm et al., 2012). The enlarged basal disk phenotype was interpreted as a result of induced differentiation of the FoxO-deficient cells. These effects in *H. vulgaris* were then proposed to be a model for the onset of aging, because FoxO silencing increased the population of terminally differentiated cells, and the loss of stem cells has been linked to aging in many species (Boehm et al., 2012).

These experiments completed by Boehm et al. (2012) showed that a reduction in FoxO activity could be detected as a large foot phenotype in the non-aging *H. vulgaris*. We find that aging *H. oligactis* show the same large foot phenotype, indicating that FoxO may be less active in the foot region after aging has been induced. The large foot phenotype appears to intensify when *H. oligactis* are incubated at 10˚ C for longer periods of time, suggesting a continuing reduction in FoxO activity as aging proceeds. These changes should be quantified in future experiments. This could be done by measuring the area of the basal disk, which would be facilitated by staining that detects peroxidase/catalase activities characteristic of basal disk cells (Hoffmeister & Schaller, 1985). Additionally, levels of FoxO expression in the basal disk region could be quantified using qRT-PCR (Boehm et al., 2012).

These results provide evidence that changes in the ISS pathway occur during aging in *H. oligactis*. The expression pattern of HTK7 changes when *H. oligactis* are induced to age, and a phenotype associated with reduced FoxO activity is observed. However, many questions remain. Comparison of the transcriptomes of non-aging and aging *H. oligactis* would provide comprehensive information about changes in expression of ISS pathway genes during aging. Work is being done currently to develop a protocol for the generation of transgenic *H. oligactis*, which would allow knockdown and overexpression of genes in the ISS pathway.

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