FoxO is a critical regulator of stem cell maintenance in immortal Hydra

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Hydra’s unlimited life span has long attracted attention from natural scientists. The reason for that phenomenon is the indefinite self-renewal capacity of its stem cells. The underlying molecular mechanisms have yet to be explored. Here, by comparing the transcriptomes of Hydra’s stem cells followed by functional analysis using transgenic polyps, we identified the transcription factor forkhead box O (FoxO) as one of the critical drivers of this continuous self-renewal. FoxO overexpression increased interstitial stem cell and progenitor cell proliferation and activated stem cell genes in terminally differentiated somatic cells. FoxO down-regulation led to an increase in the number of terminally differentiated cells, resulting in a drastically reduced population growth rate. In addition, it caused down-regulation of stem cell genes and antimicrobial peptide (AMP) expression. These findings contribute to a molecular understanding of Hydra’s immortality, indicative of an evolutionarily conserved role of FoxO in controlling longevity from Hydra to humans, and have implications for understanding cellular aging.

Results

FoxO Expression Correlates with the Undifferentiated Stem Cell State. When analyzing the stem cell signatures of the three stem cell lineages in Hydra, foxO emerged as an obvious candidate for controlling stem cell self-renewal as it is highly expressed in all three stem cell lineages (Fig. 2 A and B). To independently confirm

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foxO expression in all three stem cell lineages, we performed an additional cell-sorting experiment followed by quantitative real-time PCR (qRT-PCR) (Fig. 2C). Further independent support for this conclusion comes from mechanical separation of ectodermal and endodermal tissue followed by qRT-PCR (Fig. 2D) as well as by in situ hybridization (Fig. 2F and G). Interestingly, foxO is down-regulated upon terminal differentiation in both head and foot tissue (Fig. 2E and F) as well as in differentiating gametes within gonads (Fig. 2H), suggesting potential FoxO functions in stem cell regulation. Similar to all other invertebrate species, the Hydra magnipapillata genome contains only a single FoxO gene that, according to phylogenetic analyses (Fig. 2I and Fig. S1), groups in a basal position to all bilaterian FoxO genes, including human foxO3a, Caenorhabditis elegans dauer-formation DAF-16/foxO, and Drosophila dFOXO.

**Overexpression of foxO in Interstitial Stem Cell Lineage Increases Stem Cell and Progenitor Cell Proliferation and Induces Expression of Stemness Genes in Terminally Differentiated Nematocysts.** To examine whether FoxO plays essential regulatory roles in the processes that influence interstitial stem cell number and function, we generated two independent lines of polyps containing an actin-driven foxO-eGFP transgene (Fig. 3A) expressed in interstitial stem cells and nematoblast precursors (Fig. 3B–D).

qRT-PCR results confirm that levels of foxO transcripts are significantly elevated in polyps of both foxO-eGFP transgenic lines, compared with eGFP-expressing control polyps (Fig. 3E). Confocal microscopy of polyps overexpressing foxO in the interstitial cell lineage (Fig. 3F–I) and in ectodermal epithelial cells (Fig. S2) shows that FoxO-eGFP fusion protein is localized in both nucleus and cytoplasm of stem cells (Fig. 3F and Fig. S2A). In terminally differentiated cells in tentacles and hypostome, FoxO-eGFP localization is mostly cytoplasmic (Fig. 3B and C). To assess the role of FoxO in controlling interstitial cell proliferation, we determined the 5-bromo-2′-deoxyuridine (BrdU)-labeling index of interstitial stem cells and nematoblast precursors. Fig. 3F indicates that foxO overexpression in the interstitial cell lineage is accompanied by a significant increase in proliferation of interstitial stem cells and nematoblast precursors, which results in...
an increased number of interstitial stem cells and nematoblast precursors per polyp (Fig. 3G).

As shown previously (19–22), interstitial stem cells express cnwi and cnvas1 whereas nematoblast precursors specifically express the minicollagen mc17. We therefore asked next if foxO overexpression in interstitial cells could affect the expression of stem cell genes. qRT-PCR results show that the level cnvas1 and cnwi expression is substantially increased in polyps of both transgenic lines overexpressing foxO in their interstitial stem cells (Fig. 3H). As assessed by double in situ hybridization in polyps transfected with the control construct, nematocyte precursors express minicollagen mc17 whereas interstitial stem cells express cnwi (Fig. 3I–K). Mature nematocytes never express cnwi (Fig. 3L). In transgenic polyps overexpressing foxO in the interstitial cell lineage, we observe not only a higher density of nematoblast progenitor cells (Fig. 3M), but also a large number of nematoblast precursors expressing both cnwi and mc17 simultaneously (Fig. 3M–O). Most strikingly, cnwi transcripts can even be
found in terminally differentiated nematocytes such as stenoteles or isorhizas in both transgenic lines (Figs. 3P and Fig. S3). Thus, overexpression of foxO appears to induce expression of stem cell genes in terminally differentiated somatic cells. This finding resembles earlier observations in *C. elegans* that ectopic expression of *DAF-16/foxO* in somatic tissue also induces the ectopic expression of germ-line genes (23) and may indicate that *foxO* overexpression transfers stem cell character to terminally differentiated cells in the interstitial cell lineage.

**FoxO Silencing in Epithelial Cells Results in Enhanced Terminal Differentiation and a Slow Growth Phenotype.** To further investigate functions of FoxO in Hydra stem cells, we knocked down its expression in ectodermal and endodermal epithelial cells using a stable hairpin transcription approach (Fig. 4A). We have analyzed six independent transgenic lines (three endodermal, two ectodermal, and one ecto- and endodermal). Levels of *foxO* transcript are significantly and specifically reduced in all lines as assessed by qRT-PCR (Fig. 4B and Fig. S4). *foxO* silencing is associated with a severe enlargement of the foot and stalk region, as assessed by in situ staining with the stalk-specific Pedibin gene (24) (Fig. 4C–F and Fig. S5A). By examining the expression patterns of endodermal transcription factors known to be important for foot formation, we observed that both *nk2 homeobox* (nk2) and erythroblast transformation-specific 2 (ets2) are strongly expressed in the enlarged stalk structures (Fig. S5B). Measuring of the expression field of *pedibin* revealed (Fig. 4G) that all six transgenic lines have an increased number of *pedibin*-expressing cells, suggesting that silencing FoxO function results in an increase of terminally differentiated foot cells. The finding that *mc17*-expressing progenitor cells, which normally are never present in foot tissue, are absent from the enlarged stalks of transgenic polyps (Fig. S5B) supports this view. *foxO* silencing and the associated increase in terminally differentiated foot cells are accompanied by defects in growth rate. Quantification of growth rate under standard culture conditions shows that polyps with *foxO*-

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**Fig. 4.** *foxO* down-regulation. (A) Construct used for *foxO* down-regulation. (B) *foxO* expression levels in endodermal tissue layer of *foxO*-endo polyps, ectodermal tissue layer of *foxO*-ecto polyps, and total tissue of *foxO*-ecto/endo polyps, analyzed by qRT-PCR (*n* = 3 replicates). Asterisks indicate significant changes in expression levels (*t* test); *P* values: endo A6 line = 0.0001, endo D11a line = 0.0008, endo E1 line = 0.0032, ecto D11a line = 0.0123, and ecto/endo line = 0.0008. (C–F) In situ hybridization of *pedibin* in *foxO*-endo, *foxO*-ecto, *foxO*-ecto/endo, and control polyps. (G) Foot length compared with body length in *foxO* and control polyps, as determined by *pedibin* labeling (*n* = 10 replicates & five polyps each) and (*I*) time of bud development (*n* = 10 replicates) of *foxO*-*endo* (A6 line) and control polyps. Asterisks indicate significant differences in growth (*t* test); *P* value: <0.0001. (*H*) Growth curves (*n* = 10 replicates & five polyps each) and (*I*) time of bud development (*n* = 10 replicates) of *foxO*-*endo* (A6 line) and control polyps. Asterisks indicate significant differences in expression levels (*t* test); *P* value: <0.0001. (*J*) Expression levels of stem cell genes in *foxO*-ecto/endo polyps analyzed by qRT-PCR (*n* = 3 replicates). Asterisks indicate significant changes in expression levels (*t* test); *P* values: all <0.0001. (K) Expression levels of AMPs in *foxO*-endo polyps analyzed by qRT-PCR (*n* = 3 replicates). Asterisks indicate significant changes in expression levels (*t* test); *P* values: *hydramacin* A6 line = 0.0001, *periculin2bA6 line* = 0.0316, *hydramacinD11a line* = 0.0031, *hydramacinC1 line* = 0.0011, and *periculin2bC1 line* = 0.0136.
deficient endoderm have a reduced population growth rate, with a doubling time of 6 d compared with 4 d for the control transgenic line (Fig. 4H) due to a 2-d prolonged bud attachment to the mother polyp (Fig. 4I).

As indicated above (Fig. 2B), genes strongly expressed in all three stem cell lineages include cnwi and cnvas1. We therefore wondered if FoxO is required for the expression of these genes in epithelial tissue. As shown in Fig. 4J, in polyps with foxO-silenced ectoderm and endoderm, both cnvas1 and cnwi are significantly down-regulated. These findings indicate once more that cnvas1 and cnwi are FoxO target genes and resemble earlier observations in C. elegans (23). Taken together, these observations raise the possibility that foxO-deficient epithelial cells are driven into terminal differentiation, indicating that FoxO function is necessary for stem cell self-renewal and continuous growth.

Silencing FoxO Activity in Epithelial Cells Causes Severe Changes in the Functionality of Hydra’s Innate Immune System. Our findings are intriguing in light of prior data showing that FoxO plays a key role in controlling life span and stress resistance in both C. elegans and flies (10, 11, 13). Because previous studies reported drastic changes of the immune system with age (“immuno senescence”; for review see ref. 25), we tested whether silencing of foxO might also cause changes in the immune status of polyps by comparing the expression patterns of genes known to play important roles in Hydra’s immune system, which is predominantly located in the endoderm (2, 26–30). foxO down-regulation in endodermal epithelial cells of three independent lines reduces the expression of the AMP gene arminin and causes severe overexpression of the AMPs hydramacin and periculin2b (Fig. 4K). Analysis of the hydramacin, periculbin2b, and arminin promoters reveals the presence of numerous FoxO-binding sites (Fig. S6), indicating that FoxO can directly bind to these regulatory regions, acting either as repressor or as transcriptional activator (31). Because AMPs are key players in Hydra’s innate immunity and not only important defense molecules but also regulators of the host–microbe interaction (28, 32), our observations indicate that down-regulation of foxO results in significant changes in the functionality of Hydra’s innate immune system. This is consistent with prior results from Drosophila (33) that showed that AMP genes are activated in response to nuclear DFOXO activity. Thus, in addition to its importance in stem cell homeostasis, FoxO appears to have an unexpected role in controlling innate immune genes in Hydra.

Discussion

By the following three lines of evidence we have established that FoxO controls stem cell behavior in Hydra (summarized in Fig. S7A and schematized in Fig. S7B): (i) foxO is strongly expressed in all three stem cell lineages; (ii) overexpression of foxO in interstitial stem cells stimulates stem cell and progenitor cell proliferation and confers stemness by activation of stem cell genes to terminally differentiated cells such as nematocytes; and (iii) silencing of foxO in epithelial cells increases the number of terminally differentiated foot cells at the cost of growth rate and causes down-regulation of stem cell genes as well as changes in expression of genes controlling the functionality of the innate immune system. Based on the findings in Hydra we propose a general model in which FoxO plays a key role in controlling longevity (Fig. 5). According to this model, in immortal Hydra the high expression of foxO in all three stem cell lineages is crucial for the continuous self-renewal capacity and unlimited life span as well as the continuous maintenance of the functionality of the innate immune system. In contrast, the aging process of most organisms is caused by a reduction of foxO activity, which decreases functions of the immune system and stem cells. Aging in stem cell systems from flies to humans is associated with progressive loss of stem cell number and activity (23).

Experimental evidence in flies, worms, and mice indicates that changes in life span indeed can occur through changes in foxO expression. Mutations in daf/foxO in C. elegans prevent the longevity effect whereas overexpression increases it (23). Moreover, specific mutations in the sequence of foxO3a significantly increase human life span (9–13, 34).

Our results also delineate a role for FoxO in innate immunity. We show that silencing FoxO activity results in significant changes in the expression of AMPs. AMPs in both plants and animals are activated through pattern recognition receptors in response to microbi-associated molecular patterns. Although our observations do not elucidate the precise genetic network responsible for FoxO-mediated AMP activation, they are consistent with previous observations in flies (33) and make a significant prediction: control of stem cell self-renewal may be tightly coupled to innate immune pathways, and aging may be the consequence of both reduced stem cell function and altered innate immune defense. This may be a common feature in plants and animals because stem cells in the shoot apical meristem of plants express high levels of a peptide controlling immune signaling and microbe interaction (35).

Taken together, studies of FoxO in Hydra have several important implications. They not only reveal FoxO as a molecular factor that has contributed to the early evolution of stem cells, but also highlight intriguing similarities between Hydra and other multicellular organisms including humans, in the mechanisms that maintain stemness (14–17) and control life span (9, 12, 13, 23, 36, 37). Thus, the work furthers our understanding of stem cell self-renewal at the beginning of animal evolution and also has implications for regenerative medicine and cellular aging.

Materials and Methods

Animals and Culture Conditions. Experiments were carried out using H. vulgaris strain AEP Animals were cultured according to standard procedures (38).

Generation of Transgenic H. vulgaris Strain AEP. foxO overexpression was achieved by generating transgenic polyps expressing foxO-eGFP transgene under the control of actin promoter. The stable knockdown of foxO was achieved by generating transgenic polyps expressing a foxO hairpin construct under control of actin promoter. As control, transgenic lines expressing eGFP in the endodermal and ectodermal epithelial cell and interstitial cell lineage, respectively, were used (3, 4). SI Materials and Methods include more information.

BrdU Labeling and Detection. For analysis of cell proliferation, animals were exposed for 6 h to BrdU (39), macerated (40), and subjected to BrdU detection (39) as described previously.
In Situ Hybridization. Gene expression analysis in foxO gain-of-function and loss-of-function strains was done by in situ hybridization and double in situ hybridization as described previously (3, 41). GenBank accession numbers of the analyzed genes can be found in SI Materials and Methods.

qRT-PCR. Gene expression analysis in wild-type and foxO gain-of-function and loss-of-function strains was analyzed by qRT-PCR using the Quanti-Tect Probe RT-PCR Kit (QIAGEN) and the 7300 real-time PCR system (AB) according to the manufacturer’s protocols (for primers see Table S1). GenBank accession numbers of the analyzed genes can be found in SI Materials and Methods.

Phylogenetic Analysis. Phylogenetic analyses were based on 31 amino acid sequences of the forkhead domain, aligned using ClustalW (42). The most parsimonious tree was found using MEGA 4 (43). Maximum-parsimony and Maximum-likelihood bootstrap values were calculated based on 1,000 replications. Bayesian posterior probabilities were calculated using MrBayes version 3.1.2 (44, 45) with two independent runs of 3,500,000 generations each, sampled every 1,000 generations with four chains. SI Materials and Methods includes more information.

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Correction

DEVELOPMENTAL BIOLOGY

The authors note that they unintentionally omitted a reference to an article by Bridge et al., who first described the presence of a single FoxO gene in Hydra. The reference should be cited on page 19698, right column, first paragraph, after lines 9–10, “according to phylogenetic analyses (Fig. 2I and Fig. S1)”. The complete reference appears below.


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