REGULAR ARTICLE

Expression of pluripotency factors in echinoderm regeneration

**Vladimir S. Mashanov · Olga R. Zueva ·
José E. García-Arrarás**

Abstract Cell dedifferentiation is an integral component of
post-traumatic regeneration in echinoderms. As dedifferen-
tiated cells become multipotent, we asked if this sponta-
neous broadening of developmental potential is associated
with the action of the same pluripotency factors (known as
Yamanaka factors) that were used to induce pluripotency in
specialized mammalian cells. In this study, we investigate
the expression of orthologs of the four Yamanaka factors
in regeneration of two different organs, the radial nerve
cord and the digestive tube, in the sea cucumber *Holothuria
glaberrima*. All four pluripotency factors are expressed in
uninjured animals, although their expression domains do
not always overlap. In regeneration, the expression levels
of the four genes were not regulated in a coordinated way,
but instead showed different dynamics for individual genes
and also were different between the radial nerve and the gut.
SoxB1, the ortholog of the mammalian Sox2, was drastically
downregulated in the regenerating intestine, suggesting that
this factor is not required for dedifferentiation/regeneration
in this organ. On the other hand, during the early post-injury
stage, *Myc*, the sea cucumber ortholog of c-Myc, was signif-
icantly upregulated in both the intestine and the radial nerve
cord and is therefore hypothesized to play a central role in
dedifferentiation/regeneration of various tissue types.

Keywords Regeneration · Dedifferentiation · Pluripotency
factors · Gene expression · Echinodermata

V. S. Mashanov (✉) · O. R. Zueva · J. E. García-Arrarás
Department of Biology, University of Puerto Rico, PO Box 70377,
San Juan, PR 00936-8377, USA
e-mail: mashanovvlad@googlemail.com

Introduction

Echinoderms have emerged as important models in which to
study cellular and molecular mechanisms of post-traumatic
tissue regeneration. These animals are capable of regen-
erating different parts of their body after various types
of injuries. An important phylogenetic position, as a sis-
ter group to chordates within the monophyletic group
Deuterostomia, makes studies on echinoderms particularly
relevant for understanding what we can learn from spon-
taneously regenerating animals to develop better treatment
options for human patients.

Dedifferentiation of specialized cells is a crucial step in
echinoderm regeneration. Upon injury, specialized cells of
mature tissues are able to simplify their phenotype, initi-
ate active mitotic divisions, and give rise to new specialized
cells. There is evidence suggesting that dedifferentiated
cells become multipotent, as they not only acquire the
ability of giving rise to the cells of their original cell
type, but can also generate other cell types. For example,
radial glial cells of the injured sea cucumber central ner-
vous system (CNS) give rise to both new glial cells and
neurons (Mashanov et al. 2013). Mesothelial cells of the
regenerating digestive tube show even deeper levels of ded-
ifferentiation and can transdifferentiate across germ layer
boundaries to produce cells of the luminal digestive epithe-
lium (Mosher 1956; Mashanov et al. 2005). Even though
the ability of differentiated cells in echinoderm tissues
to acquire a broadened developmental potential has been
known for at least a decade, the molecular mechanisms
underlying this phenomenon have remained completely
unexplored. This knowledge would not only be significant
from the point of view of fundamental biology, but could
also have an impact on the development of new therapeutic
approaches.

In mammals, including humans, the limited capacity of specialized cells to undergo in vivo dedifferentiation correlates with generally poor spontaneous regeneration (Mashanov et al. 2014a). However, as has been famously shown, forced expression of a combination of just four transcription factors (now known as the Yamanaka factors), including Sox2, Klf4, c-Myc, and Oct4 can induce transformation of differentiated mammalian fibroblasts into induced pluripotent stem (iPS) cells in vitro (Takahashi and Yamanaka 2006). In spite of the great biomedical value of these data, it has remained unknown if these four transcription factors constitute a universal recipe for reprogramming of all differentiated cell types. In particular, how close are the mechanisms of experimental in vitro reprogramming to spontaneous in vivo cell dedifferentiation in regeneration-competent animals? Forced expression of Yamanaka factors was achieved under controlled conditions in cell culture and with very low efficiency, whereas large numbers of cells are known to undergo dedifferentiation in vivo in injured tissues, and these cells remain within the tissue, being affected by the whole gamut of signals from other cells and the extracellular matrix. Further comparative studies of the two phenomena would eventually contribute to deeper understanding of the mechanisms underlying the naturally occurring in vivo dedifferentiation and promote development of more efficient approaches of iPS cell generation.

Expression of Yamanaka factors in post-traumatic regeneration has been previously described in various organs of regeneration-competent vertebrates, such as fish and amphibians (Maki et al. 2009; Christen et al. 2010; Perry et al. 2013), showing variation of expression patterns in different experimental settings. For example, Oct4 was found to be required for fin regeneration in zebrafish (Christen et al. 2010), but inhibited lens regeneration in newts (Bhavsar and Tsonis 2014). This variation can be explained in part by the fact that regeneration-competent vertebrates, in particular amphibians, might have evolved a number of taxon-specific regeneration mechanisms, which are absent in other animals (Garza-Garcia et al. 2010; Brockes and Gates 2014). Therefore, in order to build a complete picture of the role that pluripotency factors play in spontaneous in vivo dedifferentiation and regeneration, the studies should be extended to other model organisms beyond vertebrates.

Here, we describe the temporal and spatial expression patterns of homologs of the four Yamanaka factors in the sea cucumber *Holothuria glaberrima*. In order to determine whether dedifferentiation/reprogramming processes in the same species involve the same molecular mechanisms in different contexts, expression of those genes was investigated in two regenerating organs: the digestive tube and the radial nerve cord. All four genes are transcriptionally active in the adult uninjured tissues of both organ systems, although their expression domains in the digestive tube

do not always overlap. In regenerating animals, individual genes showed different expression dynamics, which was also dependent on the tissue type. Our study suggests that *SoxB1*, the sea cucumber homolog of the mammalian Sox2, is not required for cell dedifferentiation and early regeneration in the digestive tube. On the other hand, stereotypical overexpression of *Myc* in regeneration of both the gut and the radial nerve indicates that it might play an important role in post-traumatic response in various echinoderm tissues.

Materials and methods

Animal collection, maintenance, and sampling procedure

Adult individuals of the sea cucumber *Holothuria glaberrima* Selenka, 1867 were collected from the intertidal zone of Puerto Rico. The animals were kept in well-aerated seawater in indoor aquaria at room temperature. To study visceral regeneration, autotomy of internal organs (evisceration) was induced by intracoelomic injection of 2–4 ml of 0.35 M KCl. The injury paradigm in the central nervous system involved surgical transection of the midventral radial nerve cord at about the mid-body level, as described previously (Mashanov et al. 2012b, 2013, 2014b). At different time points after evisceration or nerve cord transection, the animals were anesthetized by immersion into seawater containing 0.2 % solution of chlorobutanol (Sigma) for 10–30 min. Samples of normal and regenerating tissues were excised and used for quantitative PCR (qPCR) or in situ hybridization as described below.

Sequence retrieval and analysis

The sequences of the *H. glaberrima* orthologs of Yamanaka factors were retrieved from the radial nerve reference transcriptome database (doi:10.6070/H4PN93J1) (Mashanov et al. 2014b) by local TBLASTN search run on a Bio-Linux (Field et al. 2006) (http://environmentalomics.org/bio-linux/) workstation. Protein domains were identified by Pfam (http://pfam.xfam.org) and Interpro (https://www.ebi.ac.uk/interpro/) database search.

Phylogenetic analysis

Phylogenetic trees were constructed to determine the phylogenetic relationships between the sea cucumber genes and their homologs from other animals. The reference homologous sequences (listed in Electronic Supplementary Material, Table S1) were retrieved from the Uniprot and NCBI's nr databases by BLAST search with *H. glaberrima* translated ORF sequences. Multiple sequence alignments were performed with ClustalW. These alignments then served as

input to construct phylogenetic trees with MEGA software (version 5 or 6) (Tamura et al. 2013) using the neighbor-joining method and 2,000 bootstrap replicates.

Quantitative real-time PCR (qPCR)

Quantitative real-time PCR was used to determine relative expression levels of each of the four transcription factors in normal and regenerating sea cucumbers. In order to prevent possible RNA degradation, all tissue sampling manipulations were performed as quickly as possible. From non-eviscerated animals, small pieces of about the same size and wet weight were taken from each of the five regions (i.e., esophagus, three regions of the intestine proper, and the cloaca) of the digestive tube and then combined together prior to RNA extraction to represent the normal gut. From regenerating animals, the entire gut rudiment was used as the source of total RNA. Both normal and regenerating digestive tubes were sampled together with the supporting mesentery. To extract RNA from the radial nerve cord, we followed the same sampling protocol as in our previous studies (Mashanov et al. 2012b, 2014b). Briefly, the tissue samples consisted of the region of the injury gap measuring 3–4 mm across plus ∼3 mm of the flanking stump regions on either side of the wound. Pieces of similar size were also excised from uninjured animals to represent the normal radial nerve. During dissection, every effort was made to surgically separate the radial nerve cord from the surrounding tissues. After excision, the tissue samples were immediately homogenized in TRIzol reagent (Sigma). Total RNA was extracted following the manufacturer's instructions and then treated with DNAse I (Qiagen). The RNA samples isolated from the normal and regenerating digestive tube were directly used in the first-strand cDNA synthesis reaction. In the case of the RNA samples derived from the radial nerve cord, we found out that we had to perform an extra step of poly(A) RNA purification using the Poly(A)Purist kit (Ambion), since the samples contained some inhibitors of downstream reactions, which could not be removed even after repeated rounds of phenol-chloroform extraction and ethanol precipitation. Template cDNA was synthesized from 1 μg of total RNA (digestive tube samples) or from 250 ng of poly(A) RNA (radial nerve cord samples) with random hexamer primers and SuperScript II reverse transcriptase (Invitrogen).

PCR primers were designed using Primer Premier 5.0 software (PREMIER Biosoft International), and their sequences are shown in Electronic Supplementary Material, Table S2. qPCR reactions were performed in 25-μl volumes using Brilliant II SYBR Green QPCR Master Mix (Agilent) and were run on a Mx3005P qPCR System (Stratagene). Each cDNA sample was assayed twice (technical replicates), and each of the conditions was represented by three independent RNA samples (biological replicates). Threshold Ct value calling, melting curve analysis, and calculations of PCR efficiency were performed using MxPro QPCR software (Stratagene) supplied with the qPCR instrument. The raw data produced by qPCR assays were considered acceptable for further analysis if the difference between technical replicates was less than $0.5 \times$Ct and the slope values corresponding to PCR efficiencies was between -3.2 and -3.5 with the R^2 value above 0.98 (Nolan et al. 2006).

Statistical analysis of qPCR data was performed using the *MCMC.qpcr* R package (Matz et al. 2013; R Core Team 2014) in the "classic" mode, which uses a normalization procedure relative to "control" genes. The following control genes were used to normalize the qPCR expression values in the radial nerve cord dataset: *elongation factor 2 (EF2)*, *ribosomal protein rpL18a*, *Mn-superoxide dismutase (Sod)*, and *V-type proton ATPase 16-kDa proteolipid subunit (ATP6L)*. These genes have been previously shown as stably expressed in both normal and regenerating radial organs in a high-throughput RNAseq assay (Mashanov et al. 2012b, 2014b). To normalize the expression values obtained for the normal and regenerating digestive tube, we used *NADH dehydrogenase subunit 5* as a reference gene (Mashanov et al. 2010). *p* values were adjusted for multiple comparisons using the Benjamini & Hochberg methods implemented in the *p.adjust* function in R.

In situ hybridization

Antisense digoxigenin-labeled riboprobes for in situ hybridization were transcribed from PCR-generated DNA templates using Roche DIG-labeling mix. The sequences of the primers used to generate the templates are listed in Electronic Supplementary Material, Table S2. The identity of all probes was confirmed by Sanger sequencing of the templates. Tissue samples were immersion fixed overnight in 4 % paraformaldehyde in 0.01 PBS (pH 7.4, 1030 mOsm), cryoprotected in graded sucrose solutions and frozen in the OCT medium (Takara). In situ hybridization was performed on 10-μm-thick cryosections as described previously (Mashanov et al. 2010, 2012a, c).

Results

Identification and characterization of the *H. glaberrima* homologs of Yamanaka factors

The sequences coding for sea cucumber homologs of the vertebrate Yamanaka factors were retrieved from the *H. glaberrima* reference transcriptome database (Mashanov et al. 2014b) by tblastn search. Since this database consists

of contigs that were derived from an automatic assembly of next-generation sequencing reads, the sequences were validated by re-sequencing using Sanger technology. The verified sequences were deposited in GenBank under accession numbers KM281936–KM281939.

Oct1/2/11

Oct proteins are a group of developmentally important POU domain-containing transcription factors that recognize a specific 8 nt DNA sequence (octamer) (Tantin 2013). The vertebrate Oct3/4 is a core transcription factor that activates protein-coding genes and non-coding RNAs required for pluripotency (Shi and Jin 2010). In mammalian stem cells, Oct3/4 is co-expressed together with Oct1, a related transcription factor that is also known to be involved in maintenance of the stem cell phenotype (Maddox et al. 2012; Tantin 2013). For example, Oct1 is required for radial glia formation in the CNS and is also strongly expressed in Lgr5-positive intestinal stem cells (Kiyota et al. 2008; Maddox et al. 2012).

Relatively little is known about the roles of Oct proteins in non-mammalian animal models. Unlike in mammals, the sea urchin genome, the most thoroughly characterized echinoderm genome so far, contains a single Oct gene, which has been shown to be most closely related to the vertebrate Oct1 and Oct2 genes. In early sea urchin embryogenesis, this gene is involved in dorso-ventral axis specification (Range and Lepage 2011). The role of Oct in echinoderm regeneration has not been investigated so far.

The *H. glaberrima* ortholog of the vertebrate Oct3/4 has an open reading frame coding for a 779 amino acid-long protein, which contains both motifs characteristic of Pou transcription factor proteins, a Pou domain and a homeobox domain, separated by a 21-amino acid linker region (Fig. 1). Phylogenetic analysis (Fig. 2) shows that *H. glaberrima* Oct protein belongs to class II POU proteins and clusters together with Oct1/2 of the sea urchin *P. lividus* and Pou2f1 of the bivalve *C. gigas* to form an outgroup to vertebrate Oct1, 2, and 11. Therefore, the *H. glaberrima* protein was designated as Oct1/2/11.

Myc

Another of the four Yamanaka factors, mammalian c-Myc, is known to directly control transcription of as many as ~15 % of all protein-coding genes and therefore mediates a wide range of biological processes. In addition to c-Myc, mammals have other functionally redundant Myc genes, including N-Myc, L-Myc, and S-Myc (Mahani et al. 2013). Unlike mammals, many invertebrates, such as tunicates, amphioxus, sea urchins, and *Drosophila*, have a single Myc gene in their genome (Gallant 2006; Morita et al. 2009).

Among the many biological functions of Myc proteins, the control of growth and cell proliferation have been conserved between invertebrates and vertebrates (Gallant 2006).

The sea cucumber homolog of Myc contains a predicted 441-amino-acid-long open reading frame, which includes two conserved motifs that define Myc proteins, the N-terminal Myc domain and the C-terminal basic helix-loop-helix domain (Fig. 1). Our phylogenetic analysis (Fig. 3) showed that the *H. glaberrima* Myc did not belong to any of the vertebrate myc subfamilies, but instead clustered together with Myc proteins from urochordates, cephalochordates, echinoids, and asteroids. This cluster received strong bootstrap support and formed an outgroup to Myc proteins of vertebrates.

Klf1/2/4

Klf proteins are zinc-finger-containing transcription factors. Klf2 and Klf4 are known to inhibit growth, DNA synthesis, and cell cycle progression and are therefore regarded as tumor suppressor genes. Klf4 functions in reprogramming of iPS cells by promoting their survival through inhibition of c-Myc-induced programmed cell death. Simultaneously, c-Myc suppresses the anti-proliferation function of Klf4 (Takahashi and Yamanaka 2006; McConnell and Yang 2010).

Studies of the sea urchin genome have identified several Klf genes (Materna et al. 2006), whose functions in echinoderms remain largely unstudied. The sea cucumber homolog of Klf4 codes for a 323-amino-acid-long polypeptide with three zinc finger C2H2 motifs at the carboxy-terminal end of the protein (Fig. 1). Such organization is typical for all members of the Klf family (McConnell and Yang 2010). Phylogenetic analysis (Fig. 4) clusters the sea cucumber protein as an outgroup to the clade containing mammalian Klf1, Klf2, and Klf4 with a strong bootstrap support. Therefore, this sequence was designated as Klf1/2/4.

SoxB1

Sox genes code for a diverse family of transcription factors (26 members and 11 members in the human and sea urchin genome, respectively) (Howard-Ashby et al. 2006) that contain the high-mobility group (HMG) domain and are known to be involved in various aspects of development. Mammalian Sox2 protein has been shown to play a key role in acquisition and maintenance of pluripotency (Pevny and Placzek 2005; Takahashi and Yamanaka 2006; Lefebvre et al. 2007). Likewise, a related sea urchin gene, SoxB1, is thought to be involved in maintenance of the enteric neuronal precursors (Wei et al. 2011).

Our phylogenetic analysis (Fig. 5) shows that the sea cucumber homolog of mammalian Sox2 clusters together

Fig. 1 Diagram showing domain organization for the predicted Oct1/2/11, Myc, Klf1/2/4, and SoxB1 proteins of the sea cucumber *Holothuria glaberrima*
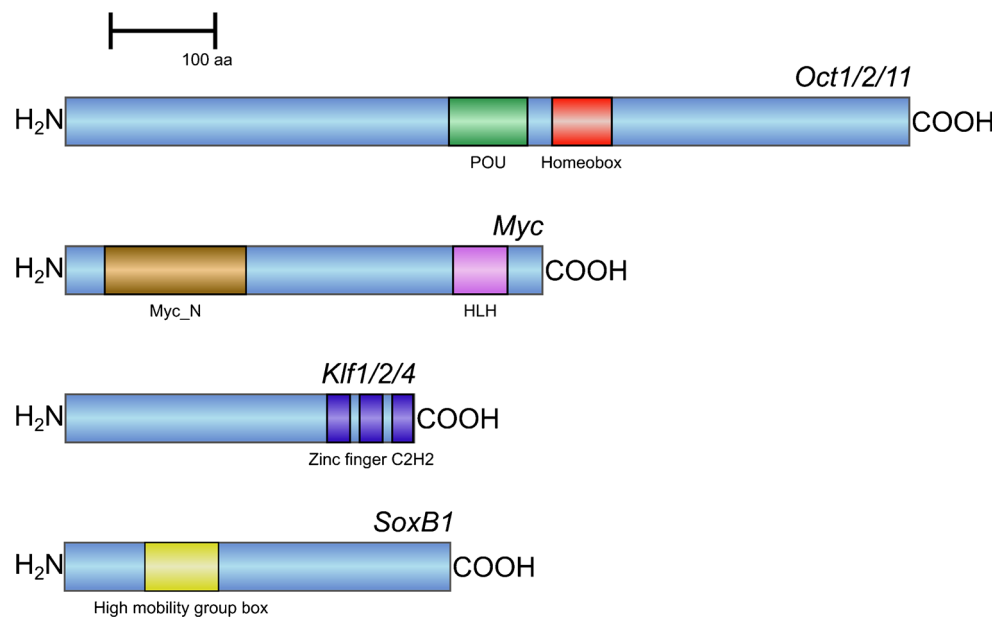
Fig. 2 Evolutionary relationships between Oct1/2/11 of *H. glaberrima* (*arrowhead*) and homologous POU proteins in other animals. The *green rectangle* indicates class II of POU transcription factors. The numbers at branch nodes indicate the percentage of recovery of the branch in replicate trees in the bootstrap test. The tree is drawn to scale. The evolutionary distances were calculated using maximum composite likelihood method and are represented as number of base substitutions per site. The database accession numbers of the sequences used in the phylogenetic analysis are listed in Electronic Supplementary Material, Table S1
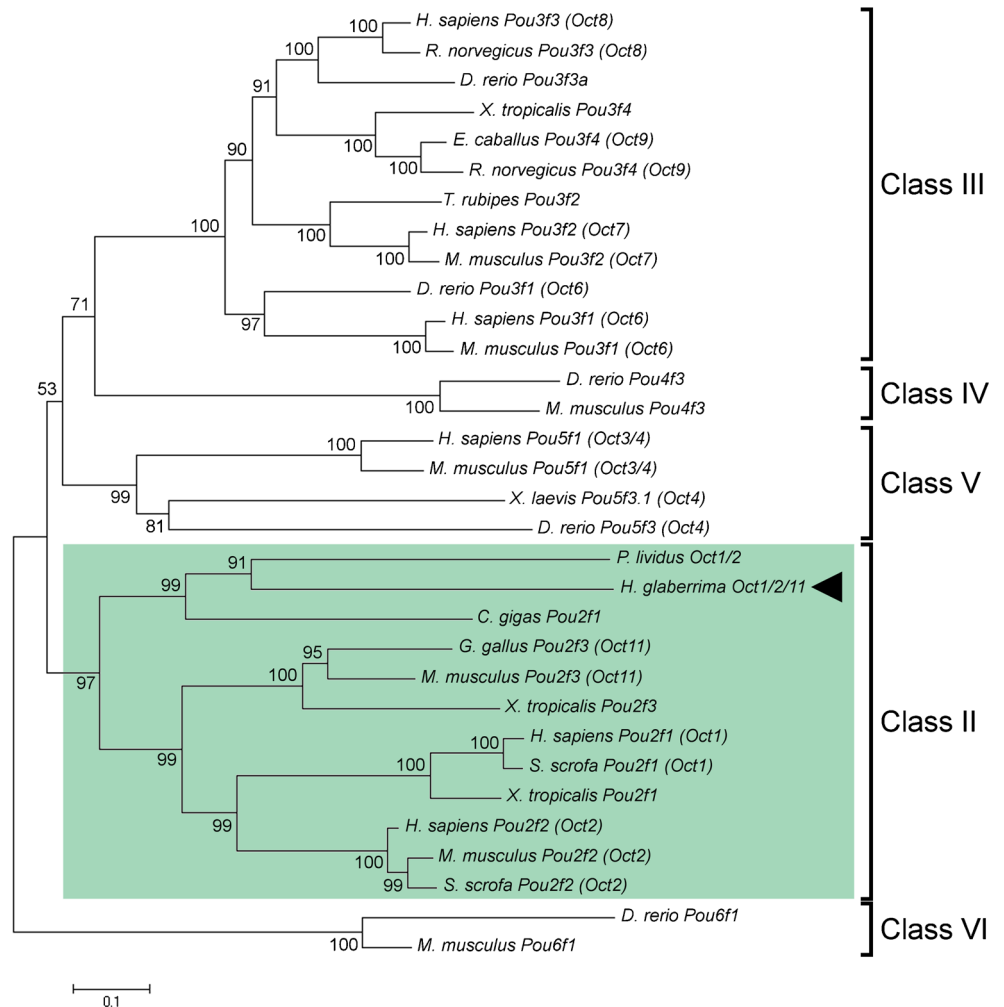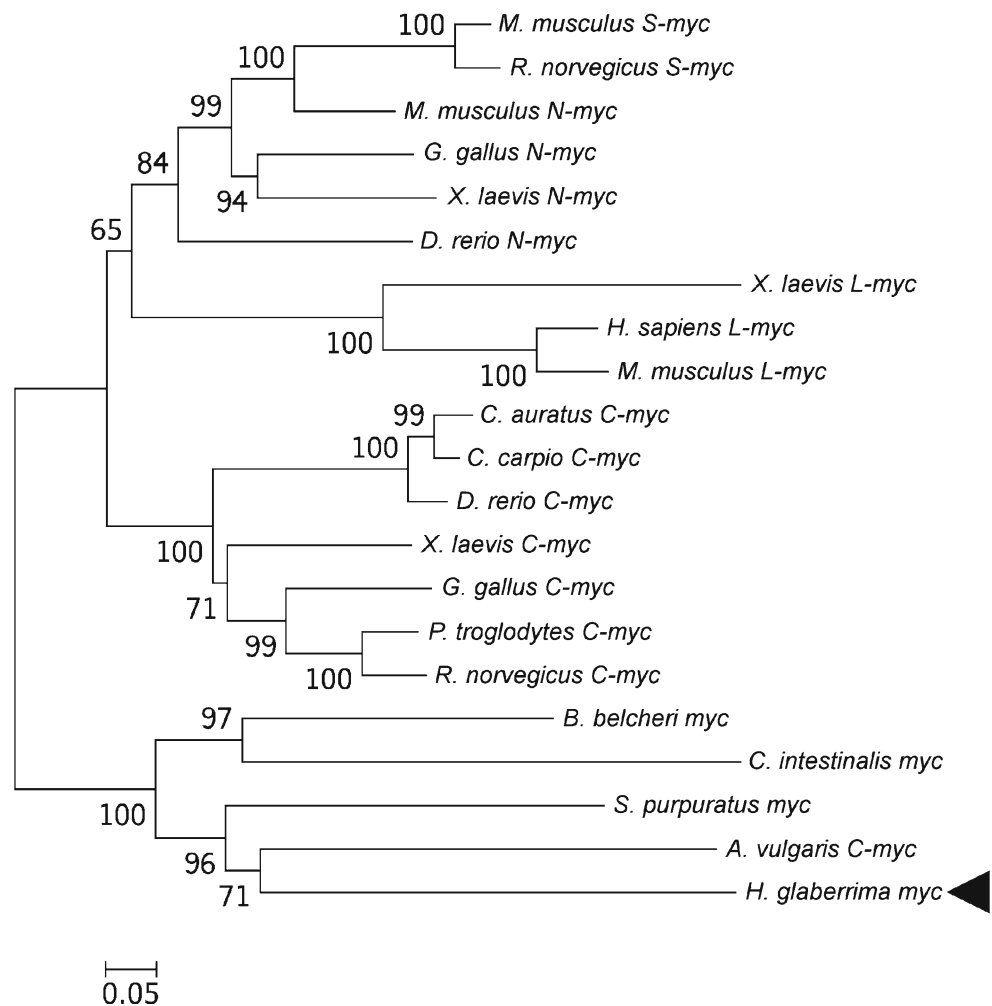
Fig. 3 Evolutionary relationships between Myc of *H. glaberrima* (*arrowhead*) and homologous proteins in other Deuterostomia

with the sea urchin SoxB1 proteins, as well as with the hemichordate protein designated as Sox1/2/3. Together, these proteins from invertebrate deuterostomes are most closely related to mammalian Sox1, Sox2, and Sox3, which constitute the so-called SoxB1 subgroup. Therefore, the *H. glaberrima* homolog of Sox2 was designated as SoxB1. This transcript codes for a 357-amino-acid-long peptide containing the signature HMG domain (Fig. 1).

**Temporal expression of *Oct1/2/11*, *Myc*, *Klf1/2/4*, and *SoxB1* in visceral and neural regeneration**

To determine if and how the sea cucumber orthologs of the mammalian pluripotency factors change their expression in the regenerating digestive and nervous systems, we ran quantitative real-time RT-PCR assays on RNA samples isolated from uninjured animals and from regenerating tissues at different time points after the injury (Fig. 6).

In the regenerating digestive tube, *SoxB1* showed a surprisingly prominent down-regulation at the early

post-evisceration stages (over 1,000-fold and ~ 13-fold on day 3 and 7, respectively). *Myc* was moderately (~3-fold) upregulated on day 3 post-evisceration, but then returned to its basal expression level, before being slightly (~1.5-fold) downregulated on day 21 post-evisceration. The two other genes, *Klf1/2/4* and *Oct 1/2/11*, were transcriptionally active in both non-autotomized and regenerating digestive tube, but did not show any significant differences in expression level across the conditions (Fig. 6a, Electronic Supplementary Material Text S3).

The expression profile of the pluripotency factors in the radial nerve cord was markedly different from that in the intestine (compare Fig. 6a and b, Electronic Supplementary Material Text S4). First, *Myc* was upregulated significantly (3–10-fold) and continuously throughout the duration of the experiment. Second, unlike in the digestive tube, *SoxB1* expression remains at normal levels in the regenerating radial nerve cord, before becoming slightly downregulated during the late regeneration phase (~1.6-fold, day 20 post-injury). Moreover, *Oct1/2/11*,

Fig. 4 Evolutionary relationships between Klf1/2/4 of *H. glaberrima* (*arrowhead*) and homologous proteins in other Deuterostomia. The tree was rooted using the Sp9 transcription factor of *Danio rerio*
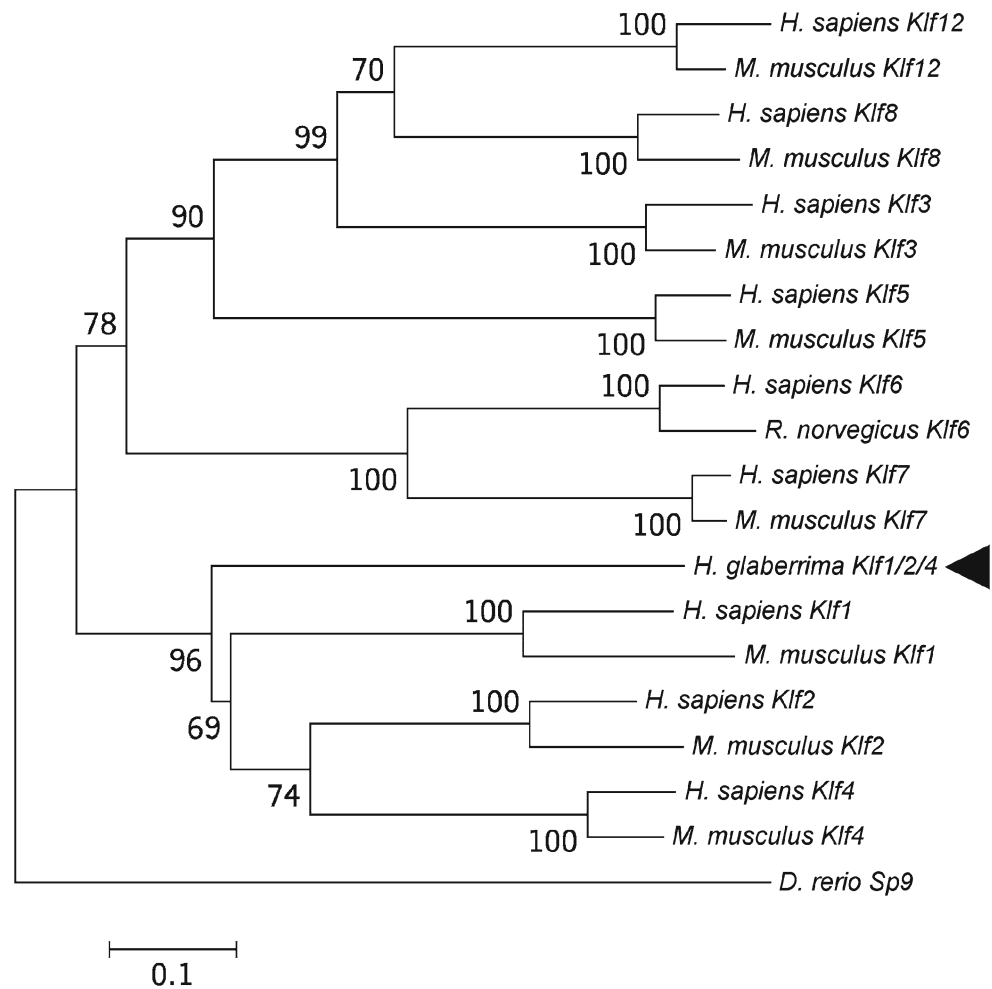
after remaining at normal levels during the early stages of neural regeneration (days 3–6), also becomes slightly down-regulated during late stages of regeneration (∼1.6-fold, days 12–21). As in the regenerating intestine, *Klf1/2/4* maintains the same level of expression both under normal conditions and during neural regeneration.

Expression of the pluripotency factors at the tissue level

Non-eviscerated digestive tube

Anatomically, the digestive tube of sea cucumbers consists of several regions including the esophagus, three subdivisions of the intestine proper (first descending intestine, ascending intestine, second descending intestine), and the cloaca (Feral and Massin 1982). Along its entire length, the digestive tube has the same basic architecture and is composed of three tissue layers: the luminal epithelium, the connective tissue partition, and the mesothelium. However, in each of those regions, the tissue components show different organization (Fig. 7a, f, k, p, u). Likewise, all four

pluripotency factors show differential expression along the length of the digestive tube.

Myc is silent in the esophagus (Fig. 7b), but extensively expressed in the luminal epithelium of the intestine proper (Fig. 7g, l, q), as well as in scattered individual cells of the mesothelium (Fig. 7l′, q′).

SoxB1, like *Myc*, is broadly expressed in the luminal epithelium of the intestine (Fig. 7h, m, r), but also in the luminal epithelium of the esophagus (Fig. 7c).

Klf1/2/4 is also strongly and broadly expressed in the luminal epithelium of the esophagus (Fig. 7d), but shows no transcriptional activity in the first descending intestine (Fig. 7i) and is very weakly expressed in the luminal epithelium of the ascending intestine (Fig. 7n). In the second descending intestine, positive in situ hybridization signal is seen in extremely rare, but strongly labeled cells of the luminal epithelium (Fig. 7s).

Oct1/2/11 is not transcriptionally active in the esophagus and ascending intestine (Fig. 7e, o), but is strongly expressed in scattered cells of the luminal epithelium in the first descending intestine (Fig. 7j) and shows very weak, but

Fig. 5 Evolutionary relationships between SoxB1 of *H. glaberrima* (*arrowhead*) and Sox proteins of other animals. *Green* and *red* rectangles indicate the SoxB1 and SoxB2 subgroups of Sox transcription factors, respectively
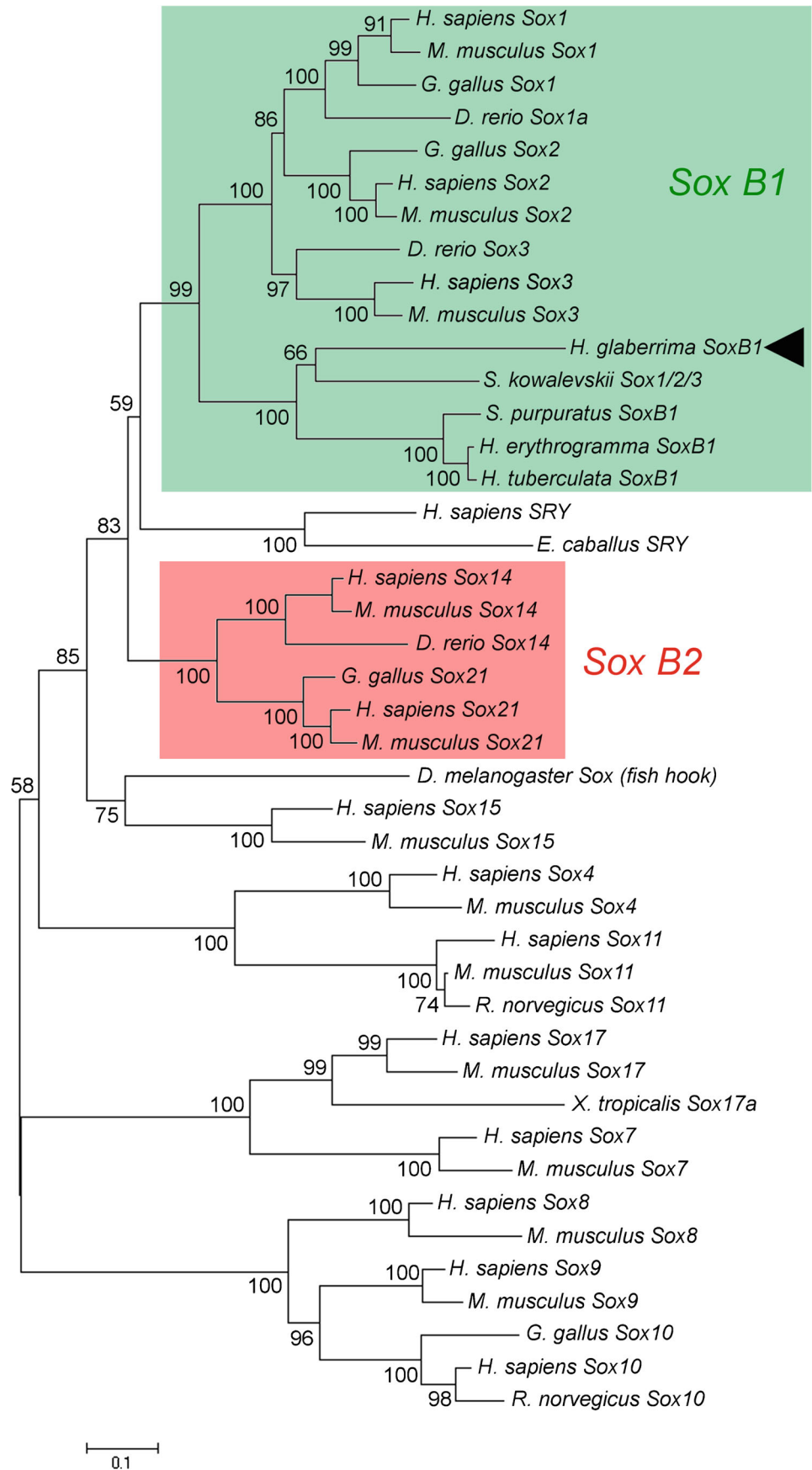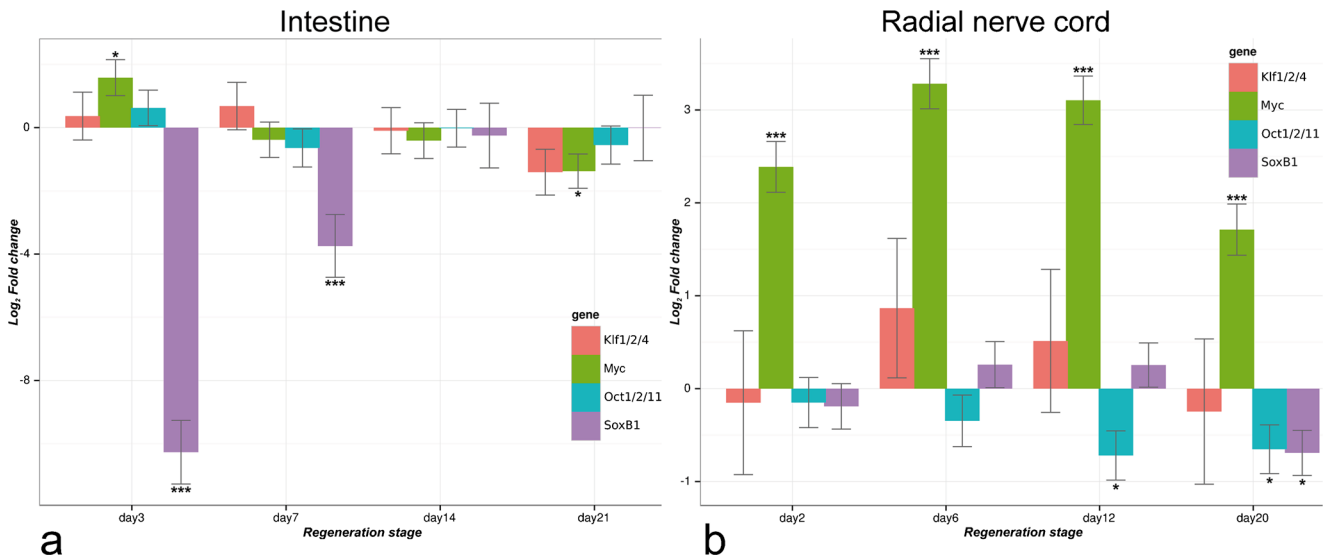
Fig. 6 Temporal expression pattern of *Klf1/2/4*, *Myc*, *Oct1/2/11*, and *SoxB1* in the regenerating digestive tube (**a**) and radial nerve cord (**b**) as determined by quantitative real time RT-PCR. Three animals were used per time point. Expression values are plotted as fold change relative to the uninjured tissues in log2 scale. Error bars show standard deviation. $^{*} p < 0.05,^{***} p < 0.001$. The full output of the MCMC.qpcr R package (Matz et al. 2013) containing relative expression values and corresponding p values can be found in Electronic Supplementary Material, Text S3, and Text S4

broad expression in the luminal epithelium of the second descending intestine (Fig. 7t).

The cloaca is the only organ of the digestive system where all four transcription factors show similar expression patterns in scattered enterocytes of the luminal epithelium (Fig. 7v, w, x, y). Three of the four pluripotency genes, *Myc*, *SoxB1*, and *Oct1/2/11*, are also broadly expressed in the cell bodies of peritoneal cells in the apical region of the cloacal mesothelium (Fig. 7v′, w′, y′).

Regenerating digestive tube

During evisceration (visceral autotomy) in *H. glaberrima*, the entire intestine detaches from the supporting mesentery and from the esophagus and cloaca at the anterior and posterior ends, respectively. The rejected organ is then expelled from the body cavity through the rupture in the cloacal wall and then through the anus (García-Arrarás et al. 1998). Subsequent regeneration involves a number of events including (a) healing of the ruptured ends of the esophagus and cloaca, (b) formation of a solid connective tissue thickening at the free margin of the supporting mesentery, and (c) ingression of the digestive epithelia of the cloaca and esophagus into this solid rudiment to restore the gut lumen. In *H. glaberrima*, the majority of the luminal epithelium of the newly regenerated intestine is derived from the cloaca, whereas the contribution of the esophagus to regeneration is limited.

The details of the anatomical organization and histological architecture of gut rudiments at different stages of visceral regeneration in *H. glaberrima* are described elsewhere (García-Arrarás et al. 1998). Here, we provide only a brief description of the anatomical structures and cellular events that are pertinent to our discussion of gene expression patterns.

On day 3 post-evisceration, dedifferentiation is initiated in the mesothelium of the free distal margin of the mesentery. At the cellular level, dedifferentiation involves remodeling of the cytoskeleton (Candelaria et al. 2006; Mashanov et al. 2005) and simplification of the epithelial architecture, but no changes are yet visible at the gross level (Fig. 8a). In line with the qRT-PCR data, there is no in situ hybridization signal for *SoxB1* on day 3 post-evisceration (Fig. 8c). The other three pluripotency factors are transcriptionally active in the mesothelium of the gut rudiment, with *Myc* being extensively expressed in most of the mesothelial cells (Fig. 8b), whereas *Klf1/2/4* and *Oct1/2/11* were detected only in some scattered cells (Fig. 8d, e).

By day 7 post-injury, the mesothelium of the free distal margin of the mesentery is completely dedifferentiated. It has the organization of a simple epithelium with no muscular component, in contrast to more proximal regions of the mesentery (Fig. 8f). This dedifferentiated mesothelium surrounds a notable swelling in the distal mesentery, which constitutes the early rudiment of the new intestine. At this stage, very weak in situ hybridization signal for *SoxB1* is detected in the mesothelium of the regenerating gut (Fig. 8h), whereas the other three transcription factors are extensively expressed in the mesothelial epithelial cells
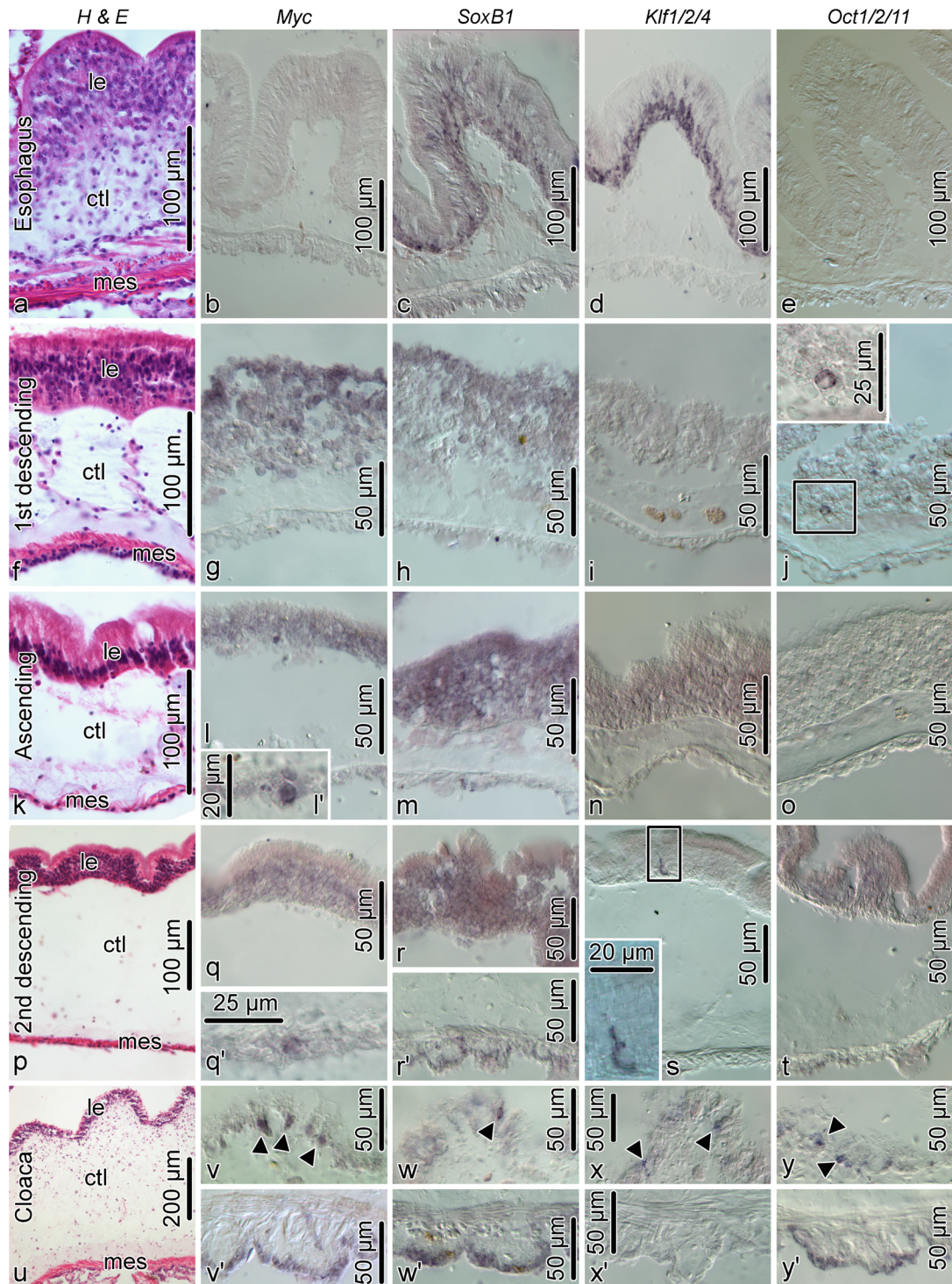
Fig. 7 Spatial expression of *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11* in the non-eviscerated digestive tube. Each horizontal row of images corresponds to a different region of the digestive tube: **a–e,** esophagus; **f–j,** first descending intestine; **k–o,** ascending intestine; **p–t,** second descending intestine; **u–y',** cloaca. The leftmost column of images (**a, f, k, p, u**) show reference paraffin sections (hematoxylin and eosin staining) for each of the regions of the digestive tube (**a,** esophagus; **f,** 1st descending intestine; **k,** ascending intestine; **p,** 2nd descending intestine; **u,** cloaca). The second through fifth columns show in situ hybridization signal with riboprobes for each of the transcripts. All micrographs show transverse sections with the luminal epithelium to the top and the mesothelium to the bottom. In cases when two micrographs are shown for a particular gut region (**l** and **l'**; **q** and **q'**; **r** and **r'**, etc.), the upper micrograph (**l, q, r,** etc) shows the luminal epithelium and the bottom micrograph (**l', q', r',** etc) shows the mesothelium. *Insets* in **j** and **s** show higher magnification views of the boxed areas. *arrowheads* indicate positively stained cells in the luminal epithelium of the cloaca. *ctl,* connective tissue layer; *le,* luminal epithelium; *mes,* mesothelium
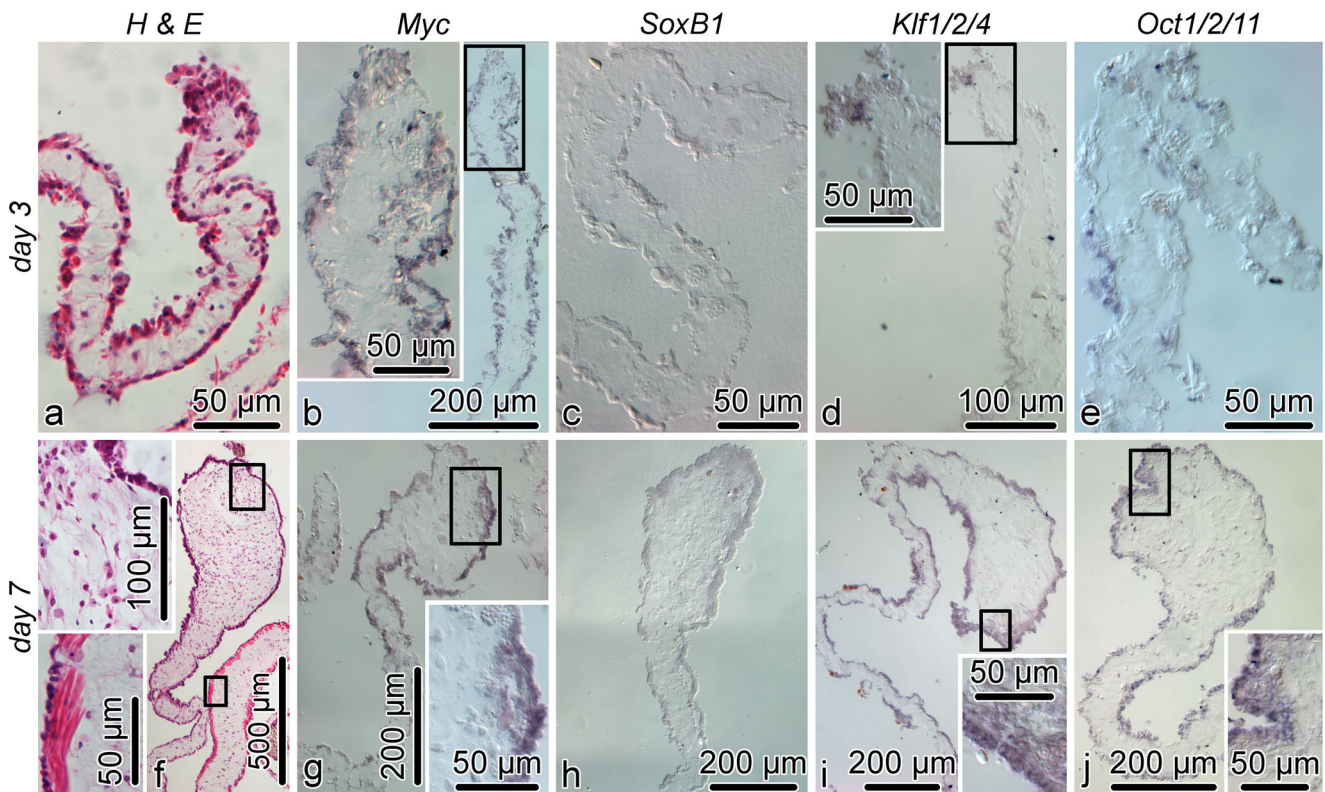
Fig. 8 Spatial expression of *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11* during early stages of visceral regeneration. The two horizontal rows of images (**a** through **e**, **f** through **j**) show cross sections of the regenerating intestine on day 3 and 7 post-evisceration, respectively. The first column (**a** and **f**) shows hematoxylin and eosin-stained reference sections. The second through fifth columns (**b–e**, **g–j**) show in situ hybridization labeling with respective riboprobes. *Insets* show high magnification view of the boxed areas in the respective main micrographs. *ctl*, connective tissue layer; *mes*, mesothelium

and also in a few mesenchymal cells directly beneath the mesothelium at the anti-mesenteric side of the developing gut rudiment (Fig. 8g, i, j).

On day 14 post-evisceration, the luminal epithelium of the cloaca starts invading the connective tissue of the solid gut rudiment (Fig. 9a). This epithelial invasion involves extensive mitotic division in the regenerating tissues (Fig. 9a′) (García-Arrarás et al. 1998). All four sea cucumber orthologs of the pluripotency factors are expressed at moderate to high levels in both the luminal epithelium and the mesothelium of the regenerating intestine. In situ hybridization signal in the luminal epithelium is often stronger than in the mesothelium (Fig. 9).

By day 21 post-evisceration, the regenerating digestive tube of *H. glaberrima* resumes its normal anatomical and histological organization (Fig. 10a), but the cells in the newly regenerated intestine are still mitotically active (Fig. 10a′), as the organ is still growing to restore its normal size and looping. All four genes are extensively expressed in most of the cells of the luminal epithelium. On the other hand, the mesothelium of the new gut shows very weak or no in situ hybridization signal (Fig. 10b–e).

None of the four transcription factors were expressed in the cells of the connective tissue partition of the gut wall.

Radial nerve cord

Five radial nerve cords (RNCs) constitute the major parts of the echinoderm central nervous system (Mashanov et al. 2006). Unlike in mammals, these regions of the echinoderm nervous system readily regenerate after injury. The key cellular event underlying this post-traumatic neural regeneration is activation of radial glial cells in the vicinity of the wound. These cells undergo dedifferentiation, form an early glial scaffold that constitutes the rudiment of the regenerating segment of the radial nerve cord, and extensively proliferate to give rise to new glial cells and, most importantly, new neurons (Mashanov et al. 2008, 2013).

Unlike in the digestive tube, the spatial distribution of the four pluripotency factors was found to be similar in both the uninjured and regenerating radial nerve cord (Fig. 11). In the normal RNC, the genes are expressed in the apical region of both the ectoneural and hyponeural neuroepithelia (Fig. 11b–e). *Myc* is also expressed in scattered cell bodies
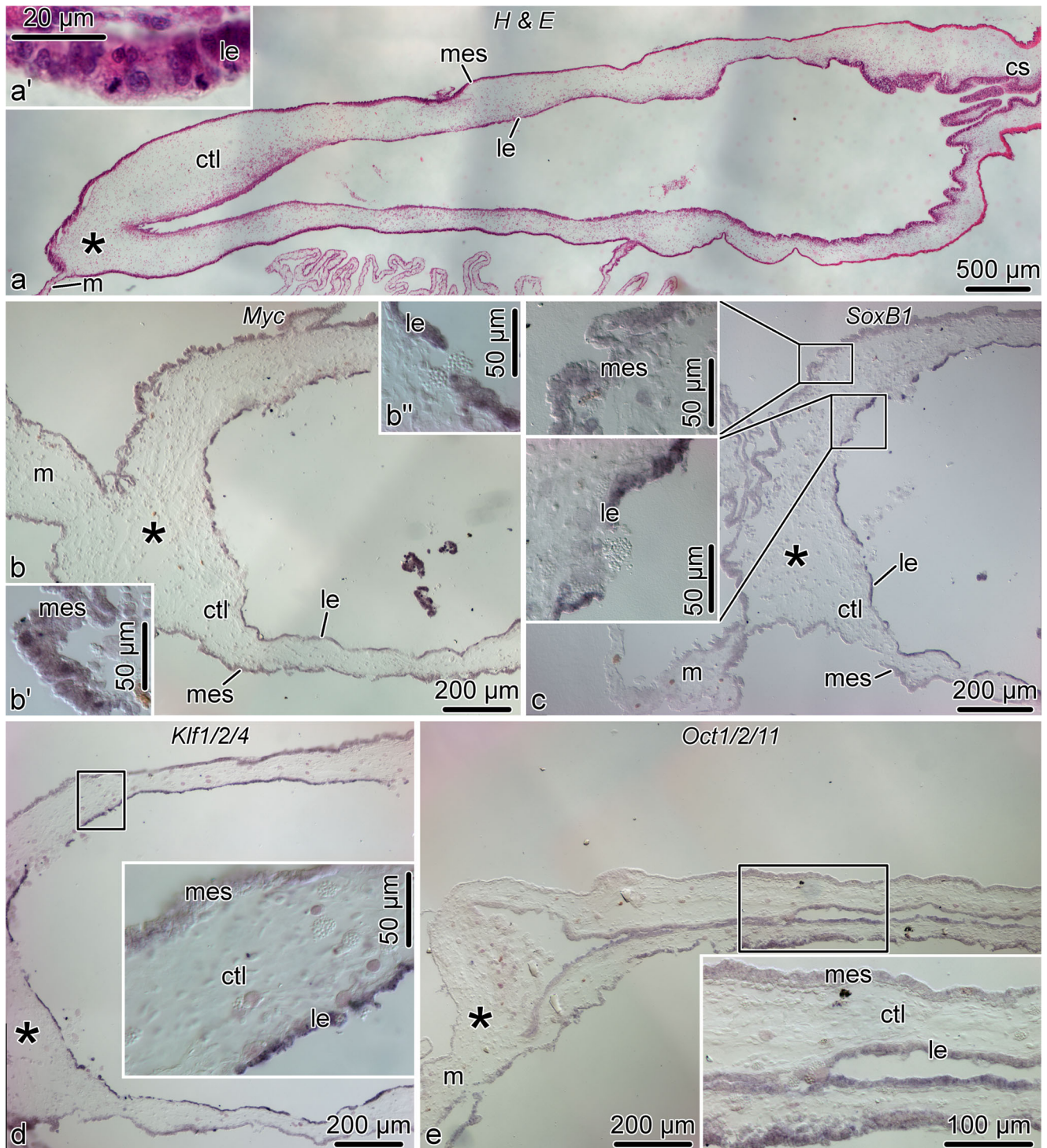
Fig. 9 Spatial expression of *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11* in the regenerating intestine on day 14 post-evisceration. All micrographs show longitudinal sections of the regenerating intestine. The growing anterior tip of the gut rudiment (*asterisk*) is on the left, whereas the cloacal stump (*cs*) is on the right. **a**, reference section stained with hematoxylin and eosin. **a′**, high-magnification view of the luminal epithelium of the growing intestinal rudiment showing abundant mitotic figures. **b**, *Myc* expression in the intestinal rudiment. **b′** and **b″** show high-magnification views of the mesothelium and luminal epithelium, respectively. **c**, **d**, and **e** show expression of *SoxB1*, *Klf1/2/4*, and *Oct1/2/11*, respectively. *Insets* in **c–e** are high magnification views of the boxed areas in the respective main micrographs. *cs*, cloacal stump; *ctl*, connective tissue layer; *le*, luminal epithelium; *m*, mesentery; *mes*, mesothelium
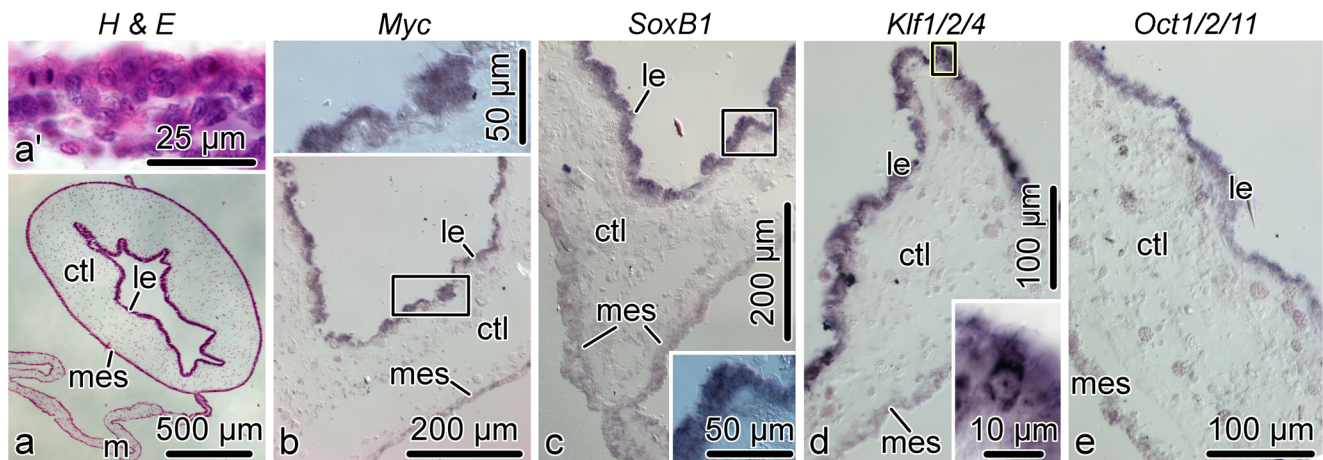
Fig. 10 Expression of *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11* in the tissues of the newly regenerated intestine on day 21 post-evisceration. All micrographs are cross sections. **a** Low-magnification view of the newly regenerated intestine; reference paraffin section stained with hematoxylin and eosin. **a**′ high-magnification view of the luminal epithelium showing mitotic figures. **b–e** In situ hybridization with riboprobes for *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11*, respectively. *Insets* show high magnification views of the boxed areas in the respective main micrographs. *ctl* connective tissue layer; *le* luminal epithelium; *m* mesentery; *mes* mesothelium

throughout the neural parenchyma (Fig. 11b′). During regeneration, the four genes continue to be transcriptionally active in the apical regions of the neuroepithelia of the stumps (Fig. 11g–o), but are also expressed in glial tubes, which constitute the early rudiments growing across the wound gap (Fig. 11k–o′). After the wound gap has been bridged, all four genes continue to be expressed in the newly created region of the RNC (Fig. 11q–t).

Discussion

In this study, we investigated expression of homologs of the four Yamanaka factors in spontaneous post-traumatic regeneration of two different body parts, the digestive tube and the central nervous system, in the sea cucumber *H. glaberrima*. Previous morphological studies (Candelaria et al. 2006; Mashanov et al. 2005, 2008, 2013) indicated that regeneration in both of these organs is preceded by extensive dedifferentiation of specialized cells. Dedifferentiating cells undergo activation of nuclear heterochromatin, cytoskeleton reorganization, and complete or partial loss of their specialized cytoplasmic features. Once dedifferentiation is completed, the cells contribute to regeneration through extensive cell division. Their progeny undergo cell migration and rearrange themselves to reconstruct the lost body part. The available data indicate that the dedifferentiated cells may become at least transiently multipotent, as they are able to give rise not only to new cells of the same cell type, but also to other cell types. For example, dedifferentiated mesothelial cells can transdifferentiate into enterocytes of the gut luminal epithelium (Mashanov et al. 2005), whereas radial glial cells of

the regenerating central nervous system generate neurons (Mashanov et al. 2013). Therefore, we wanted to see if the four pluripotency Yamanaka factors were expressed in spontaneously regenerating tissues, whose specialized cells undergo dedifferentiation to broaden their developmental potential.

Our results show that dedifferentiation of specialized tissues and subsequent regeneration in *H. glaberrima* do not involve coordinated upregulation of all four pluripotency factors. For example, *Klf1/2/4* and *Oct1/2/11* remained expressed at about the same level as in the normal tissues. Moreover, those genes that change their expression do so in different ways, depending on the tissue type, suggesting existence of multiple gene regulatory scenarios operating in different contexts in the same species. The most notable difference between the gut and the RNC is the absence of *SoxB1* expression during early stages of intestinal regeneration, whereas it remains expressed at the normal level in the injured radial nerve. This observation suggests that cell dedifferentiation in the gut mesothelium does not require expression of *SoxB1*, which is puzzling, as Sox2, the homolog of *H. glaberrima* SoxB1, is not merely expressed in various vertebrate regeneration models (Maki et al. 2009; Christen et al. 2010; Perry et al. 2013; Luz-Madrigal et al. 2014), but is absolutely required for regeneration (Christen et al. 2010). The functional significance of *SoxB1* down-regulation in the regenerating sea cucumber intestine is unknown and can only be determined in future experiments involving forced ectopic over-expression.

One of the common features between the regenerating gut and CNS in *H. glaberrima* is significant upregulation of *Myc* during early post-injury stages, when both organs
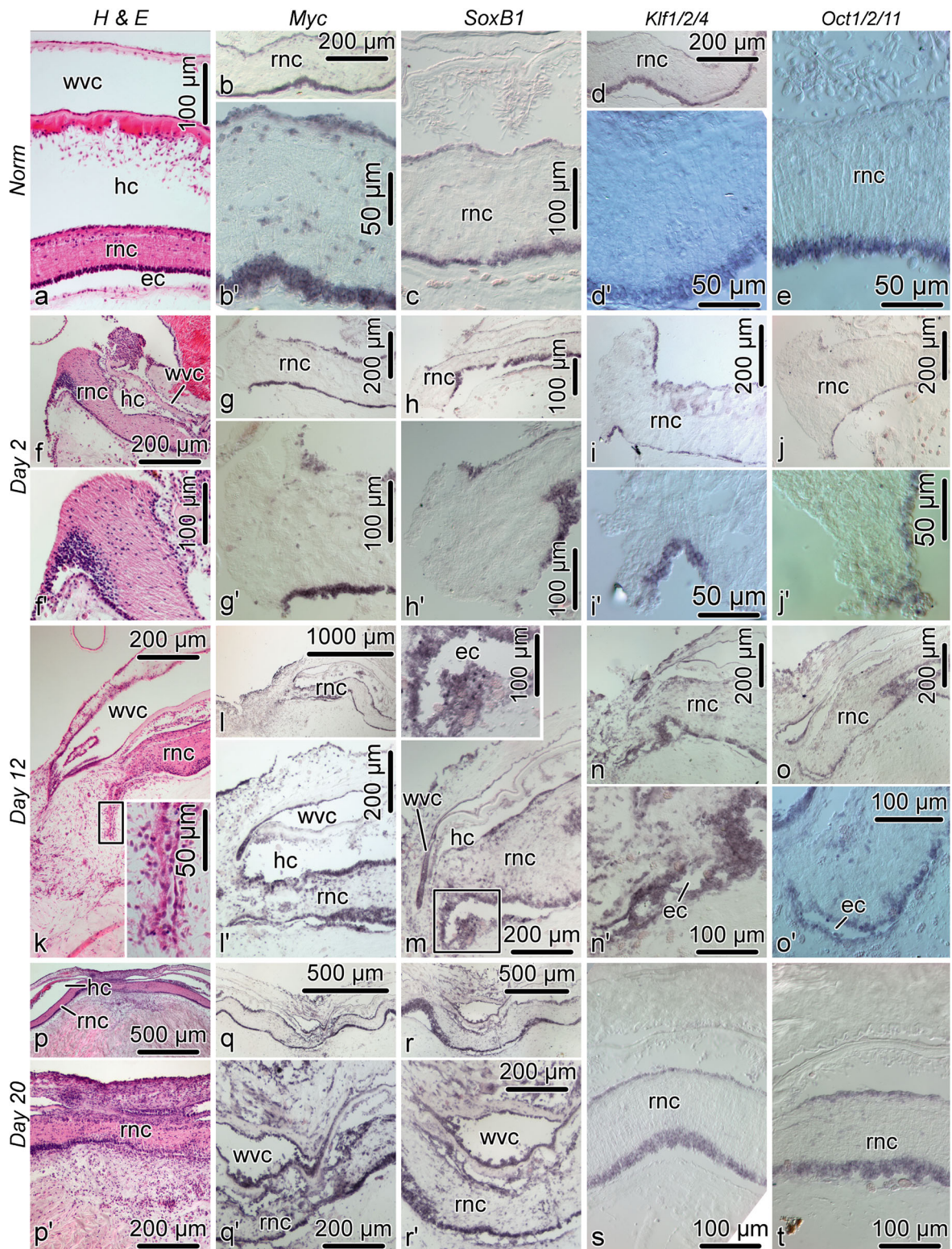
Fig. 11 Expression of *Myc*, *SoxB1*, *Klf1/2/4*, and *Oct1/2/11* in the uninjured and regenerating radial nerve cord. All sections are longitudinal. Horizontal rows of images correspond to the normal animals and three different stages of regeneration. The first vertical column shows reference paraffin sections stained with hematoxylin and eosin. The second through fifth columns show in situ hybridization labeling with respective riboprobes. *Insets* in k and m show high magnification view of growing glial tubes (boxed areas in the respective main micrographs). When two micrographs are shown for a particular condition (e.g., **b** and **b′**, the upper (e.g., **b**) and lower (e.g., **b′**) images show an overview and high-magnification view, respectively). *ec* epineural canal; *en* ectoneural neuroepithelium; *hc* hyponeural canal; *hn* hyponeural neuroepithelium; *wvc* water-vacular canal; *rnc*, radial nerve cord

undergo extensive cell dedifferentiation (Candelaria et al. 2006; Mashanov et al. 2013). Myc proteins play a central role in dedifferentiation and regeneration even in poorly regenerating mammals. For example, it has been shown that expression of c-Myc was required for regeneration of the intestinal epithelium (Ashton et al. 2010). In the mammalian CNS, experimental forced expression of v-Myc, a viral homolog of c-Myc, stabilized the undifferentiated state of embryonic radial glia. When injected into the injured adult spinal cord, these cells contributed to increased neuroprotection and axonal regrowth (Hasegawa et al. 2005). Therefore, our observations combined with known roles of Myc proteins in regeneration and control of cell differentiation in various organisms (Mahani et al. 2013) make the sea cucumber *Myc* gene a promising candidate for further studies. Our ongoing efforts are aimed at determining the functional role of *Myc* over-expression in dedifferentiating tissues of *H. glaberrima*.

The fact that some pluripotency factors are present in the regenerating sea cucumber tissues and may even be significantly up-regulated in response to injury (e.g., *Myc*), whereas other factors (such as *SoxB1*) are absent and obviously not involved in regeneration, suggests that even though there might be some parallels between in vivo dedifferentiation and in vitro iPS cell generation, the mechanisms are not exactly the same. One possible explanation why these two processes can be different is that the cells that dedifferentiate in vivo, unlike iPS cells, may not be pluripotent, but at best only multipotent (Maki et al. 2009; Christen et al. 2010). Alternatively, we cannot rule out that other, yet unknown, factors can contribute to in vivo dedifferentiation in regeneration-competent animals.

Conclusions

In the sea cucumber *H. glaberrima*, the orthologs of mammalian Yamanaka pluripotency factors, *Myc*, *SoxB1*, *Oct1/2/11*, and *Klf1/2/4*, are expressed in the adult uninjured central nervous system and in the digestive tube, although in the latter, their expression domains do not overlap (except in the cloaca).

Expression of individual pluripotency factors is not coordinated in injured/regenerating tissues. Some of these genes (*Oct1/2/11* and *Klf1/2/4*) keep being expressed at about the same levels as in uninjured animals, while others (*SoxB1* and *Myc*) undergo considerable changes in expression.

There are differences in pluripotency factor expression dynamics between the regenerating radial nerve cord and the regenerating intestine, suggesting that cell dedifferentiation in different regenerating organs may involve organ- and/or tissue-specific mechanisms.

Drastic down-regulation of *SoxB1* in the regenerating intestine during the early post-evisceration phase suggests that expression of this gene is not required for cell dedifferentiation in this organ.

Consistent up-regulation of *Myc* in both intestinal and neural regeneration in *H. glaberrima* suggests that this gene may play an important role in dedifferentiation/regeneration in various tissues and organs.

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