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Expression of stem cell factors in the adult sea cucumber digestive tube

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Abstract

Homeostatic cell turnover has been extensively characterized in mammals. In their adult tissues, lost or aging differentiated cells are replenished by a self-renewing cohort of stem cells. The stem cells have been particularly well studied in the intestine and are clearly identified by the expression of marker genes including *Lgr5* and *Bmi1*. It is, however, unknown if the established principles of tissue renewal learned from mammals would be operating in non-mammalian systems. Here, we study homeostatic cell turnover in the sea cucumber digestive tube, the organ with high tissue plasticity even in adult animals. Both the luminal epithelium and mesothelium express orthologs of mammalian *Lgr5* and *Bmi1*. However, unlike in mammals, there is no segregation of these positively labeled cells to specific regions in the luminal epithelium, where most of the cell proliferation would take place. In the mesothelium, the cells expressing the stem cell markers are tentatively identified as peritoneocytes. There are significant differences among the five anatomical gut regions in cell renewal dynamics and stem factor expression. The cloaca differs from the rest of the digestive tube as the region with the highest expression of the *Lgr5* ortholog, lowest level of *Bmi1*, and the longest retention of BrdU-labeled cells.

Keywords

Cell turnover; intestinal stem cells; *Lgr5*; *Bmi1*; echinoderm; sea cucumber

1. Introduction

Homeostatic cell turnover is a common strategy to maintain tissue integrity in long-lived multicellular animals. This strategy typically involves segregation of the tissue into two cohorts of cells. Most cells are post-mitotic, fully differentiated and have a limited lifespan,

6. Author Contribution

VM, OZ, and JEGA conceived the study. VM and OZ carried out the experimental procedures. VM and DM performed sequence analysis and phylogenetic analysis. All authors interpreted the results. VM drafted the manuscript. All authors read and finalized the manuscript.

8. Conflict of interests

The authors declare that they have no conflict of interest

whereas the second groups of cells – stem cell progenitors – retain the capacity to self-renew and replenish the tissue that constantly sheds worn-out differentiated cells.

One of the best characterized models of homeostatic cell turnover is the digestive epithelium of the mammalian small intestine. This epithelial tissue is organized as a series of repeated villus-crypt units. The villi are composed of mature specialized cell types of limited life span, whereas the crypt contains stem cells that replenish the continuously dying mature cells of the villus (Beumer and Clevers, 2016). There are two distinct stem cell populations in the mammalian intestinal crypt: frequently dividing *Lgr5*-expressing cells and less numerous and less frequently dividing *Bmi1*-expressing cells (Barker et al., 2007; Schepers et al., 2011; Yan et al., 2012; Li et al., 2016; Lund and Verzi, 2016). One functional distinction between those two stem cell cohorts might be that the *Lgr5*⁺-cells are mostly involved in maintaining the homeostatic cell turnover of the intestinal epithelial cells under basal conditions, whereas the *Bmi1*⁺-cells are normally quiescent, but can be activated by injury to replenish the more vulnerable *Lgr5*⁺-cells (Yan et al., 2012).

The two marker genes – *Lgr5* and *Bmi1* – that distinguish the two intestinal stem cell types were found to be implicated in different aspects of stem cell maintenance. *Lgr5*, leucine-rich-repeat-containing G-protein-coupled receptor, is an R-spondin receptor, which, upon binding to its ligand, enhances Wnt signal by repressing the Wnt negative feedback loop (Glinka et al., 2011; de Lau et al., 2014). *Lgr5* is itself a Wnt target gene (de Lau et al., 2014). The *Lgr5*⁺ stem cells of the mammalian intestinal epithelium therefore themselves depend on Wnt3 which is produced by nearby Paneth cells (Flanagan et al., 2015; Sailaja et al., 2016). *Bmi1* (B-cell-specific Maloney murine virus integration site) is a major component of the polycomb repressor complex that modules epigenetic gene silencing. It facilitates stem cell maintenance and self-renewal in a transcription-dependent manner (Siddique and Saleem, 2012).

Since *Lgr5* and *Bmi1* are now the bona fide standard genetic markers of the intestinal stem cells in mammals, we asked if they would be implicated in tissue renewal in non-mammalian systems. Sea cucumbers, and echinoderms in general, are known for the plasticity of their adult tissues (Franco et al., 2013; Mashanov et al., 2014a; Ben Khadra et al., 2015). One manifestation of this plasticity is the capacity to autotomize (eviscerate) and then quickly regrow the digestive tube and associated visceral structures. The microanatomical changes, cellular phenomena, and molecular processes associated with this dramatic post-traumatic tissue regrowth have been best studied in the Caribbean brown rock sea cucumber *Holothuria glaberrima* (reviewed in Mashanov and García-Arrarás (2011) and Mashanov et al. (2014a)). This species autotomizes almost all of its digestive tube, with the exception of the two short terminal regions – the esophagus and cloaca – at the anterior and posterior ends of the body (see Additional File 1 for anatomical reference). The cells in the two stumps will then proliferate and form the anterior and posterior gut rudiments, respectively, whose lumina will grow towards each other and eventually fuse to generate a new anatomically continuous digestive tube (García-Arrarás et al., 1998). Non-eviscerated adult sea cucumbers also gradually replace cells in the gut wall (Mashanov and García-Arrarás, 2011) (Additional File 1, C'), however this homeostatic cell turnover has never been properly characterized. Our question here is twofold. First, we asked if the patterns of

physiological cell renewal would be the same in the “permanent” terminal regions (i.e., esophagus and cloaca, the regions that harbor high regenerative potential) as opposed to the “transient” regions that can be discarded and regrowth. Second, we seek to compare the pattern of homeostatic cell turnover between echinoderms and mammals to get the first insight into evolution of tissue maintenance mechanisms in deuterostomes.

Here, we show that the digestive epithelium and mesothelium, i.e. the inner and outer epithelial layers of the sea cucumber digestive tube, contain cells that express orthologs of the mammalian stem cell markers *Lgr5* and *Bmi1*. Unlike in mammals though, neither the cells expressing the stem cell markers, nor BrdU-incorporating cells are restricted to any particular region within the epithelia. The rate of cell proliferation was similar across the five anatomical regions of the digestive tube, however, the labeled cell progeny persisted significantly longer in the cloaca than in any other region. The two terminal regions – esophagus and cloaca – clearly stood out as the tissues with the highest expression of the *Lgr5* ortholog.

2. Materials and Methods

2.1. Animal collection and maintenance

Adult individuals of *Holothuria glaberrima* (Selenka, 1867) were hand collected during low tide from the intertidal area of northeast Puerto Rico. The animals were then kept in aerated natural seawater in indoor tanks at room temperature. The water was changed weekly. Before dissection, the animals were anesthetized in 0.2% chlorobutanol dissolved in seawater.

2.2. BrdU incorporation assay

The cells that have undergone cell divisions were identified by their ability to retain the 5-bromo-2'-deoxyuridine (BrdU) label in their chromosomal DNA. Previous experiments (García-Arrarás et al., 1998) showed that a single intraperitoneal injection followed by a short chase period labels only very rare cells in the non-eviscerated digestive tube. In order to increase the number of labeled cells, we performed saturating BrdU injections, which were repeated every 12 hours over the course of four days. Each animal, therefore, received a total of eight injections. BrdU (Sigma-Aldrich) was dissolved in PBS and was injected into the main body cavity at the dosage of 50 mg/kg using an insulin syringe. The animals were sacrificed at two different time intervals – 12 hours (referred to below as 0 weeks) and 8 weeks – after the last BrdU injection. Five animals were used at each time point.

Tissue samples were processed for BrdU immunohistochemistry following the procedure described elsewhere (Mashanov et al., 2015b). Pieces of each of the five anatomical regions of the digestive tube were fixed overnight in buffered (0.01 M PBS, pH 7.4) 4% paraformaldehyde. The tissue samples were then washed in the buffer, cryoprotected in sucrose and embedded in the OCT compound (Sakura). Serial cryosections were cut at 10 μ m and collected on gelatin-covered slides. Every third section, five sections per animal, was immunostained for further analysis. The sections were washed in PBS, followed by 0.5% Triton X-100 and then subjected to an acid treatment with 2N HCl for 30 min at 37 °C. The

sections were blocked in 2% goat serum and then incubated in the primary anti-BrdU antibody (1:400, GenWay, 20-783-71418) for 16 hours at 4 °C. Following extensive washes, the secondary antibody (FITC-conjugated Goat Anti-Rat, 1:50, GenWay, 25-787-278232) was applied for 1 hour at room temperature. The nuclei were stained with propidium iodide. The sections were then mounted in an antifading medium containing 10% Mowiol 4-88 (Calbiochem) and 2.5% DABCO (Sigma-Aldrich).

The immunostained sections were photographed with the Nikon Eclipse Ni epifluorescent microscope using a 40× objective, and the cells were then counted on digital micrographs using the Cell Counter plugin in the Fiji image analysis software (Schindelin et al., 2012). All statistical computations were performed in R (R Core Team, 2015). The data were found to deviate from normal distribution. They were therefore analyzed using the generalized linear modeling approach with quasipoisson error distribution. The R code that was used in computations and the sample output are available in Additional File 2.

2.3. Sequence retrieval and analysis

The sequences corresponding to the sea cucumber orthologs of *Bmi1*, *Lgr5*, and *Wnt3* were identified in the reference transcriptome of *H. glaberrima* (Mashanov et al., 2014b) by running TBLASTN search with the mouse proteins as query sequences. Protein domains were identified by Pfam (Finn et al., 2016) and InterPro (Finn et al., 2017) database search.

To confirm gene orthology, a phylogenetic analysis was performed to establish the evolutionary relationship between the *H. glaberrima* genes and homologous proteins from other animals. These homologous sequences were retrieved by BLAST searches against the UniProt (<http://www.uniprot.org>), NCBI nr and EchinoBase (<http://www.echinobase.org>) databases. Their accession numbers are listed in Additional File 3.

Sequence alignments were generated by the ClustalW and Muscle modules in the MEGA software (Tamura et al., 2013) and then checked manually. Phylogenetic trees were constructed using the neighbor-joining method with 2,000 bootstrap replicates.

2.4. Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real-time PCR was performed as previously described (Mashanov et al., 2015a) to determine the relative abundance of transcripts in the five anatomical regions of the sea cucumber digestive tube. Briefly, RNA was extracted from small tissue samples (not exceeding 100 mg) dissected from four different *H. glaberrima* individuals (biological replicates). Template cDNA was synthesized with Random Hexamer Primers and SuperScript II Reverse Transcriptase (Invitrogen) following the manufacturer's protocol. PCR primers were designed with Primer Premier 5.0 (PREMIER Biosoft) and their sequences are shown in Additional File 4. qRT-PCR reactions were set up using Brilliant II SYBR Green QPCR Master Mix (Agilent) following the manufacturer's instructions and then run on a Mx3005P qPCR system (Stratagene) for 40 cycles followed by melting curve analysis. Each RNA sample was assayed twice (technical replicates). The qRT-PCR data were considered acceptable, if the difference between the technical replicates did not exceed $0.5 \times C_t$ and the slope values corresponding to the PCR efficiencies fell within the range of -3.2 to -3.5 with R^2 above 0.98 (Nolan et al., 2006). Statistical analysis of the expression

data was performed with the *MCMC.qpcr* R package (Matz et al., 2013) in the “informed” mode using the *NADH dehydrogenase subunit 5* as the reference gene. The raw expression data and PCR efficiency values are available in a spreadsheet format in Additional File 5. The R code used to process the data and the corresponding output can be found in Additional File 6.

2.5. In situ hybridization

DIG-labeled riboprobes were transcribed from PCR-generated templates. The sequences of the PCR primers are listed in Additional File 4. The tissue samples were fixed overnight in a fixative containing 4% paraformaldehyde in 0.01 M PBS. They were then cryoprotected and sectioned at 10 μ m. The cryosections were collected on TESPA-covered (Sigma-Aldrich A3648) 12 mm round glass coverslips (EMS 72196-12). Chromogenic *in situ* hybridization reactions were carried out in 24-well sterile tissue culture plates as previously described elsewhere (Mashanov et al., 2012).

3. Results

3.1. Cell renewal

To characterize the homeostatic cell renewal in the sea cucumber digestive tube, we subjected normal adult individuals of *H. glaberrima* to intracoelomic injections of the thymidine analog BrdU. Previous studies (García-Arrarás et al., 1998) showed that dividing cells in the *H. glaberrima* gut are slow cycling and need to be exposed to a DNA synthesis marker for extended periods of time to be labeled in sufficiently high numbers. We therefore administered BrdU in a series of eight BrdU injections (50 mg/kg) over 4 days to target those slow cycling cells. The injected animals were divided into two groups, five individuals in each, which were sacrificed 12 hours (this group was designated as 0 weeks) and 8 weeks after the last BrdU injection, respectively. The shorter chase period provides a “snapshot” of the cell renewal patterns and identifies the regions within the tissues where the proliferating progenitors reside. The long chase period, on the other hand, provides an insight into how long the newly generated progeny of the dividing cells persist in the tissue. This approach identifies “label-retaining cells” that are capable of surviving for extended periods of time after having undergone cell division and are either post-mitotic or divide infrequently (Hamilton and Henry, 2016).

The animals that were examined shortly after the last BrdU injection (0 weeks chase period) did not show any significant difference in the abundance of labeled cells across the five anatomical regions of the digestive tube in any of the gut wall tissue layers (Fig. 1, Additional File 7, Additional File 8). In contrast, after a chase period of 8 weeks, the cloaca clearly stood out as the gut region where labeling-retaining cells were most abundant in all three tissue layers, whereas the esophagus contained the lowest number of BrdU⁺-cells. The three anatomical regions of the intestine (first descending, ascending, and second descending) showed intermediate abundance of labeled cells.

An analysis of how the abundance of BrdU⁺-cells changes with time in each of the gut anatomical regions (Fig. 2) shows a significant drop (~2.5–5-fold) in the number of labeled

cells in the anterior regions of the digestive tube, including the luminal epithelium and mesothelium of the esophagus and the luminal epithelium of the first descending intestine. In contrast, the abundance of labeled cells did not change significantly with time in the two posterior regions of the intestine (the ascending and the second descending intestine), and increased (~2.5 fold) in the connective tissue layer of the cloaca.

3.2. Identification of the sea cucumber homologs of stem cell factors

The homologous relationship of the sea cucumber genes with their counterparts in other deuterostomes was established by both the phylogenetic analysis and by checking for the appropriate protein domain organization.

Bmi1—In our phylogenetic analysis (Fig 3), the sea cucumber *Bmi1* clusters together with homologous sequences from other invertebrate deuterostomes and these proteins together are most closely related to the vertebrate *Bmi1* and its paralog *Pcgf2* (*Mel-18*). The *H. glaberrima* *Bmi1* homolog has an open reading frame coding for a 440 amino acid-long protein, which contains two characteristic domains (Fig 6). The N-terminal C3HC4 RING finger domain is known to be critical for interactions between *Bmi1* and other nuclear proteins (Hemenway et al., 1998; Sanchez-Pulido et al., 2008). The second domain, with a yet unknown function, is the recently discovered RAWUL motif, which is a typical feature of Polycomb group repressor complex I proteins (Sanchez-Pulido et al., 2008).

Lgr5—Phylogenetic analysis shows that the sea cucumber *Lgr5* homolog clusters together with similar sequences from other invertebrate deuterostomes to form an outgroup to the vertebrate *Lgr4*, *Lgr5*, and *Lgr6* (Fig 4). The *H. glaberrima* protein was therefore designated as *Lgr4/5/6*. The open reading frame of this protein codes for a 978 amino acid long protein. The domain architecture analysis shows all hallmarks of class B Lgr proteins (Fig 6), which in mammals includes *Lgr4*, *Lgr5*, and *Lgr6* (Van Hiel et al., 2012; de Lau et al., 2014). The sea cucumber *Lgr4/5/6* contains a characteristically large number — fourteen — of leucine-rich repeat units (LRR) that are known to form a large extracellular domain. Another typical feature is the presence of a C-terminal seven-transmembrane domain (7TM).

The *H. glaberrima* *Wnt3* predicted protein contains 370 amino acids (Fig 6). Phylogenetic analysis clusters its coding sequence together with *Wnt3* sequences from other deuterostomes with high bootstrap support (Fig 5).

3.3. Bmi1 and Lgr4/5/6 are unevenly expressed across the anatomical regions of the digestive tube

Expression of the sea cucumber *Bmi1*, *Lgr4/5/6*, and *Wnt3* was studied at the bulk tissue level with quantitative real-time PCR (qRT-PCR) and at the cell level with *in situ* hybridization. The results of qRT-PCR are shown in a graphical form in (Fig 7) and the raw data are available in Additional File 5. Expression of *Bmi1* was significantly (8–16 fold) lower in the cloaca than in any of the more anterior regions of the digestive tube. In contrast, *Lgr4/5/6* shows a clear bimodal distribution with one distinct peak in the esophagus and another one in the cloaca. Between the two terminal gut regions, *Lgr4/5/6* transcripts are significantly (~5-fold) more abundant in the cloaca than in the esophagus. *Wnt3* was

detected throughout the digestive tube and did not show any significant variation in the levels of expression across different regions. In situ hybridization corroborated the qRT-PCR data. The strongest and most extensive positive staining with the *Lgr4/5/6* probe was seen in both the luminal (digestive) epithelium and in the mesothelium of the cloaca (Fig. 9a). The positively stained luminal epithelial cells were numerous and did not show any obvious organized distribution pattern and were interspersed among the *Lgr4/5/6*-negative cells. In the mesothelium, the positively stained cells occupied the apical region of the epithelium (i.e., facing the coelom), which corresponds to the position of the cell bodies of peritoneal cells (Mashanov et al., 2004). In the esophagus, the strongly labeled cells (presumably peritoneocytes) are equally abundant in the mesothelium (Fig. 8a'), whereas in the luminal epithelium, the *Lgr4/5/6*-positive cells are rare, albeit strongly labeled (Fig. 8a). In agreement with the qRT-PCR data, *in situ* staining for *Lgr4/5/6* is the weakest in the first descending region of the intestine with only very rare singly scattered cells detected in the luminal epithelium and none in the mesothelium (Fig. 8b). The ascending and second descending regions both showed intermediate levels of staining with the *Lgr4/5/6* riboprobe (Fig. 8c, d). In the digestive epithelium of these intestinal regions, most cells are moderately labeled, but some show noticeably stronger hybridization signal. The exact reason for this variation in *Lgr4/5/6* expression at the individual cell level is yet unknown, although it does suggest a certain level of molecular and probably functional heterogeneity among intestinal enterocytes. In the mesothelium, very few lightly labeled cells are present in the ascending intestine and none in the second descending intestine.

Bmi1 is moderately to strongly expressed in many, although not all, cells in the digestive epithelium of all regions of the digestive tube except for the cloaca (Fig. 8e–h, 9b, b'). Again, the biological significance of this variation in expression levels among enterocytes within the same gut region remains to be established. In the cloacal digestive epithelium, its expression is also widespread, although the labeling intensity is much weaker (Fig. 9b). In the mesothelium, moderate to weak expression is seen in the presumably peritoneal cells of the esophagus, the first descending intestine, and cloaca (Fig. 8e, f; Fig. 9b').

Since the cloaca is the gut region with the highest levels of *Lgr4/5/6* expression, we decided to investigate the expression pattern of *Wnt3*, a signaling protein required for maintenance of *Lgr5*⁺ cells in mammals (Beumer and Clevers, 2016). It was moderately to strongly expressed in numerous scattered cells of both the digestive epithelium and mesothelium (Fig. 9c – c''). Of note, unlike *Lgr4/5/6* and *Bmi1*, the mesothelial expression of *Wnt3* is not limited to the cells at the apical surface (presumably peritoneocytes), but is also often seen in deeper cells (myocytes and/or neurons) (Fig. 9c'').

4. Discussion

4.1. The sea cucumber luminal epithelium expresses stem cell markers, but lacks specialized proliferative regions

We showed here that orthologs of two mammalian intestinal stem cell markers, *Bmi1* and *Lgr5*, are abundantly expressed in the adult digestive tube of the sea cucumber *H. glaberrima*. Since in mammals, *Lgr5*-expressing cells require *Wnt3* signaling, we also showed the presence of abundant *Wnt3*-producing cells in the cloaca – the region of the sea

cucumber digestive tube with the highest level of *Lgr4/5/6* expression. This indicates the conservation of the main molecular components of the intestinal stem cell niche even outside of the phylum Chordata. The distribution of *Bmi1* and *Lgr4/5/6*-expressing cells in the sea cucumber tissues were clearly different from the localization pattern of the intestinal stem cells in the mammalian intestine. In mammals, the mucosal luminal epithelium is organized as a highly ordered system of repeated villus-crypt units. Both *Bmi1*⁺ and *Lgr5*⁺ intestinal stem cells are restricted to the crypt, while villi are formed by several differentiated cell types (Beumer and Clevers, 2016). In contrast, the sea cucumber luminal epithelium lacks such ordered structure. Although it does form folds in all five regions of the digestive tube (Additional File 1), the folds are often irregular and are not associated with clearly defined crypt regions. The pattern of cell renewal in the sea cucumber gut is not tied to the tissue geometry. Instead, proliferating cells scattered throughout the tissue. The extensive distribution of the cells expressing the stem cell markers throughout the gut epithelial tissues mirrors the lack of modular organization. The *Lgr4/5/6*-positive and *Bmi1*-positive cells are interspersed between cells that don't express these genes. The exact reason for this type of tissue architecture in the sea cucumber digestive epithelium is unknown. One possible explanation for the lack of spatial segregation of the cells expressing the stem cell markers to restricted regions of the luminal epithelium may be the well known plasticity of differentiated cells in echinoderms. It has been shown that fully differentiated cells are capable of either partial or complete dedifferentiation, when the cell reduces or completely eliminates specialized cytoplasmic features (e.g., secretory vacuoles) and re-enters the mitotic cycle (Mashanov et al., 2014a). It is therefore possible that the cell lineage relationships between putative stem cells and differentiated progeny is bidirectional, with the specialized cells being capable of re-expressing stem cell markers.

4.2. Peritoneal cells are stem cells candidates in the gut mesothelium

The stem cell markers *Bmi1* and *Lgr4/5/6* are expressed not only in the luminal epithelium, but also in the mesothelium (the outer coelomic epithelial layer of the gut wall), most prominently in the two terminal regions of the digestive tube — the esophagus and cloaca. Importantly, within the mesothelium the expression of these two markers was clearly restricted to the cell bodies located in the apical region of the epithelium. The echinoderm visceral mesothelium is a tissue with complex architecture that includes abundant myocytes and neuronal cell bodies and their processes (García-Arrarás and Dolmatov, 2010; Mashanov and García-Arrarás, 2011). The supporting framework of the mesothelium is formed by peritoneal cells, whose cell bodies form the apical surface of the epithelium. We can therefore conclude that it is mostly the peritoneal cells that express *Bmi1* and *Lgr4/5/6* in the esophageal and cloacal mesothelium. Expression of stem cell markers in this cell type corroborates earlier observations that clearly demonstrated plasticity of this cell type in the context of post-traumatic regeneration. Peritoneal cells reacted to injury by undergoing extensive dedifferentiation and were capable of giving rise not only to other peritoneocytes, but also to muscle cells and even to the cells of the luminal epithelium and coelomic fluid (Mashanov et al., 2005; García-Arrarás and Dolmatov, 2010; Mashanov and García-Arrarás, 2011; Sharlaimova et al., 2014).

4.3. Heterogeneity of stem cell niche characteristics among the gut regions

Even though neither the expression pattern of the stem cell markers nor the pattern of BrdU incorporation show any significant spatial segregation within any of the five anatomical regions of the digestive tube, there are clear differences in both characteristics among the regions. The expression of *Lgr4/5/6* along the anterior-posterior axis of the digestive tube is clearly bimodal with the peak levels in the esophagus and cloaca and much lower levels in all three regions of the intestine. This observation correlates with the fact that the two terminal regions are retained after evisceration and contribute the cellular material for regeneration of the autotomized intestine. The difference in the stem cell status between the “permanent” terminal gut regions and the “temporary” intestine, which can be autotomized and then reconstituted, is a promising avenue for future research. Between the two terminal regions, the cloaca is characterized by the higher expression levels of *Lgr4/5/6* and also the lower levels of *Bmi1*. Further functional studies will establish if these differences in the expression of stem cell markers are linked to the distinct patterns of cell turnover and subsequent fate of the cellular progeny in the two gut regions. Our results demonstrate that even though there is no significant difference in the basal rate of cell proliferation, after a long chase period (8 weeks), many of the labeled cells disappear from the tissues of the anterior gut regions, but persist in the posterior regions. The number of BrdU-labeled cells even increases with time (~2.5-fold, from 1.6% to 3.9%) in the connective tissue of the cloaca, suggesting that some of the progeny of dividing cells give rise to migratory cells that eventually move from the epithelial tissues of the gut wall (i.e. the luminal epithelium and/or mesothelium) into the connective tissue layer.

5. Conclusions

Taken together, our results demonstrate that

- homeostatic self-renewal takes place in all five anatomical regions of the sea cucumber digestive tube
- epithelial cells in the digestive epithelium and peritoneal cells in the gut mesothelium express orthologs of stem cell gene markers *Lgr5* and *Bmi1*
- in contrast to mammals, the pattern of cell division and expression of stem cell markers are not restricted to any specific regions within the gut wall tissues
- there are significant differences among the five anatomical regions of the digestive tube in terms of tissue renewal dynamics and stem cell factor expression
- the cloaca — the terminal posterior region of the gut — clearly stands out as the region with the highest expression of *Lgr4/5/6*, lowest level of *Bmi1*, and longest retention of BrdU-labeled cells

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Abundance of BrdU-labeled cells in the five anatomical regions of the digestive tube after two chase periods. **a – d** 0 weeks (12 hours after the last BrdU injection). **e – h** 8 weeks. *Esoph*, esophagus; *I Desc*, first descending intestine; *Asc*, ascending intestine; *II Desc*, second descending intestine. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.01$

Fig. 2.

Boxplot showing differences in the abundance of BrdU-labeled cells between two chase periods, 0 weeks (0 w) and 8 weeks (8 w). **a–c** esophagus (*Esoph*); **d–f** first descending intestine (*IDesc*); **g–i** ascending intestine (*Asc*); **j–l** second descending intestine (*IIDesc*); **m–o** cloaca. * $P < 0.05$; ** $P < 0.01$

Fig. 3.

Evolutionary relationships between *Bmi1* of *H. glaberrima* (arrowhead) and related sequences from other animals. The tree was generated by neighbor-joining method. The numbers at branch nodes show the percentage of recovery of the branch in bootstrap tests. Numbers below 50% are not shown. The tree is drawn to scale. The evolutionary distances were calculated using the maximum composite likelihood method and are represented as number of base substitutions per site. The tree was rooted using the *Bmi1*-like sequence of *Hydra vulgaris*.

Fig. 4. Neighbor-joining tree representing evolutionary relationships between Lgr4/5/6 of *H. glaberrima* (arrowhead) and related sequences from other animals. The tree was rooted using the Lgr-like sequence of *Hydra vulgaris*.

Fig. 5. Evolutionary relationships between Wnt3 of *H. glaberrima* (arrowhead) and Wnt protein-coding sequences from other animals. The tree was rooted using the Wnt3-like sequence of *Hydra vulgaris*.

Fig. 6. Domain organization of the predicted *Bmi1*, *Lgr4/5/6*, and *Wnt3*. *7tm_1*, seven-transmembrane domain; *LRR*, leucine-rich repeat unit; *RAWUL*, ring finger and WD40 associated ubiquitin-like domain; *wnt*, Wnt domain; *zf-C3HC4*, C3HC4 RING finger domain.

Fig. 7.

Expression of *Lgr4/5/6*, *Bmi1*, and *Wnt3* in across the five anatomical regions of the digestive tube in *H. glaberrima* at the bulk level as determined by quantitative real-time RT-PCR. The analysis was performed on four individuals. Expression values are plotted in relative units in the log2 scale. *Esoph*, esophagus; *I Desc*, first descending intestine; *Asc*, ascending intestine; *II Desc*, second descending intestine. * $P < 0.05$; ** $P < 0.01$. The the raw C_t data, R code used in the analysis, and the full output of the *MCMC.qpcr* package (Matz et al., 2013) are available in Additional File 5 and Additional File 6.

Fig. 8. In situ hybridization. Localization of the *Lgr4/5/6* and *BmiI* transcripts by in the esophagus, first descending *I Desc*, ascending (*Asc*), and second descending (*II Desc*) regions of the digestive tube. *ctl*, connective tissue layer; *de*, digestive epithelium; *me*, mesothelium. *Arrowheads* label rare positively stained cells in the digestive epithelium in **a** and **b**.

Fig. 9. In situ hybridization. Localization of the *Lgr4/5/6*, *BmiI*, and *Wnt3* transcripts in the tissue layers of the cloaca. *ctl*, connective tissue layer; *de*, digestive epithelium; *me*, mesothelium. The insets in **a** show high magnification views of the regions of the digestive epithelium and mesothelium marked with rectangles in the main image. **b** and **c** show the digestive epithelium, whereas **b'** and **c''** show the gut mesothelium. The rectangle in **c** marks the region of the digestive epithelium, which is shown at higher magnification in **c'**.