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Identification and expression characterization of WntA during intestinal regeneration in the sea cucumber *Apostichopus japonicus*

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ABSTRACT

Wnt genes encode secreted glycoproteins that act as signaling molecules; these molecules direct cell proliferation, migration, differentiation and survival during animal development, maintenance of homeostasis and regeneration. At present, although the regeneration mechanism in *Apostichopus japonicus* has been studied, there is a little research on the Wnt signaling pathway in *A. japonicus*. To understand the potential role of the Wnt signaling pathway in *A. japonicus*, we cloned and sequenced the *WntA* gene in *A. japonicus*. Protein localization analysis showed that WntA protein was ubiquitously expressed in epidermal cells, the muscle and submucosa of the intestinal tissue. After stimulation and evisceration, the dynamic changes in expression of the *WntA* gene and protein showed that WntA was constitutively expressed during different stages of intestine regeneration in *A. japonicus*, with higher levels during the early wound healing stage and late lumen formation in the residual and nascent intestinal tissues, indicating its response to intestinal regeneration. Simultaneously, cell proliferation and apoptosis analysis showed that the patterns of cell proliferation were similar to the patterns of WntA protein expression during different intestinal regeneration stages in this organism. Taken together, these results suggested that WntA might participate in intestinal regeneration and may be connected with cell proliferation, apoptosis in different intestinal layers. This research could establish a basis for further examination of WntA functions in *A. japonicus* and *Wnt* genes in other echinoderms.

1. Introduction

Regeneration has fascinated biologists for many years. Although our understanding of regeneration has grown rapidly over the past few years, regeneration remains one of the least understood developmental processes. Regenerative potential in the animal kingdom is not uniformly distributed among the different phyla and can be highly divergent even between closely related species (Brockes and Kumar, 2008; Bely and Nyberg, 2010). In an effort to gain a better understanding of the true variety of regeneration mechanisms, large numbers of taxa should be further studied.

So far, the comprehensive studies on regeneration have been carried out in a number of species, including Hydroid (Galliot and Chera, 2010; Bosch, 2007; Traversetti et al., 2016; Gahan et al., 2016; Rodrigues et al., 2016) and planarian (Owlarn and Bartscherer, 2016) from the invertebrate system, and ascidians (Kassmer et al., 2016) and *Xenopus* (Sugiura et al., 2009) from the chordata. Nevertheless, regenerative potential is maximally present in echinoderms among the deuterostomes, which can quickly renew most injured organs, as observed in brittle stars and crinoids (García-Arrarás et al., 1998; Thorndyke et al., 2001; Patruno et al., 2002, 2003; Bannister et al., 2005; Bannister and Powell, 2008). In particular, sea cucumbers are excellent models for studying organ regeneration, since some species (including *Apostichopus japonicus*) possess a unique defense mechanism called evisceration when they undergo chemical or physical stress (García-Arrarás et al., 1998; Zheng et al., 2006; Dolmatov and Ginanova, 2009; Sun et al., 2011). The missing organs and appendages can concurrently regenerate at a reasonably rapid pace afterwards (Marushkina and Gracheva, 1978; Shukalyuk and Dolmatov, 2001).

Wnt genes encode secreted glycoproteins that act as signaling molecules which direct cell proliferation, migration, differentiation and survival during animal development, maintenance of homeostasis and regeneration (Labus et al., 1998; Hobmayer et al., 2000; Poss et al., 2000; Guder et al., 2006; Liu et al., 2008; Petersen and Reddien, 2009;

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De Robertis, 2010; Willert and Nusse, 2012; Clevers and Nusse, 2012; Clevers et al., 2014). In recent years, Wnt regulated pathways have been increasingly associated with regenerative phenomena, including blastema formation of the regenerating limbs in anuran tadpoles (Yokoyama et al., 2007), lens regeneration in newts (Hayashi et al., 2006) and bone (Kim et al., 2007), hair follicle (Ito et al., 2007) and deer antler regeneration in mammals (Mount et al., 2006). In addition, Wnt pathways have also been implicated in other invertebrate regeneration models. In planaria, Wnt signaling is necessary for proper brain pattern formation (Kobayashi et al., 2007) and regenerative requires inhibition of canonical Wnt signaling (Sikes and Newmark, 2013; Liu et al., 2013). In holothurians, the *Wnt* genes have been identified as a primary target in understanding in intestinal regeneration (WNTA, WNT1, WNT2, WNT4, Wnt5, WNT6, WNT7, WNT9, WNT10, WNT16) (Girich and Dolmatov, 2014). However, *Wnt* gene function during intestinal regeneration in echinodermata have mostly been evaluated from the third day in *Eupentacta fraudatrix* or *Holothuria glaberrima*. Expression of the *Wnt6* gene has also been evaluated from the third day in *A. japonicus* (Sun et al., 2013). However, there is limited information about *WntA* at the initial stages (before day 3) of *A. japonicus* intestinal regeneration. WntA has recently been shown to regulate multiple regeneration stages in *E. fraudatrix* (Girich and Dolmatov, 2014) and planarian (Adell et al., 2010). It has also been shown that WntA is the only Wnt gene lost during the evolution of deuterostomes, suggesting that it may play an important role in evolution (Kusserow et al., 2005). *WntA* has not been studied in *A. japonicas* previously. Therefore, we decided to evaluate the function of WntA as a potential regulator of intestinal regeneration in the sea cucumber *A. japonicus* and add to the knowledge of its potential role in evolution.

In the present study, we cloned the *WntA* gene from *A. japonicus* and studied its expression characteristics during different stages of sea cucumber intestinal regeneration. These data established a basis for further examination of *WntA* functions in *A. japonicus* and our findings could provide important information for future studies of the *Wnt* genes in other echinoderms.

2. Material and methods

2.1. Experimental animals

Adult *A. japonicas* sea cucumbers were collected from the coast of the Qingdao, Shandong Province and immediately transported to the laboratory. They were allowed to adapt to laboratory conditions for 1 week in aerated circulating seawater at 15–17 °C before experimentation. Evisceration was induced by injecting 2–5 ml of 0.35 M KCl into the coelomic cavity (Mashanov et al., 2010). Eviscerated animals were kept in well-aerated in-door seawater tanks. The well-being of animals was ensured by keeping the density of *A. japonicus* in the tanks at approximately one individual per liter seawater. All sea cucumbers were anesthetized in 6% $MgCl_2$ for about 1 h before being sacrificed (Sun et al., 2013). At least five animals were used at each regeneration stage (control, 0, 30 min, 1, 2, 6, 12 h, 3, 7 and 14 days) for studies. We began to record time from the point where the sea cucumber had expelled the entire intestine, sea cucumber without stimulation or evisceration was used as the control. Part of the residual and nascent intestinal tissues were removed under RNase-free conditions and stored in liquid nitrogen until use. Meanwhile, a second section was fixed in 4% paraformaldehyde for 20 h at 4 °C, then gradually dehydrated through a graded series of alcohol concentrations, and finally embedded in paraffin for immunohistochemical staining, cell proliferation and apoptosis experiments.

2.2. Cloning of full-length WntA cDNA

Total RNA samples were extracted from the intestinal tissue and DNase-treated using the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen), and the quality was determined by agarose gel electrophoresis and quantifies using a Nanodrop spectrophotometer (Thermo Fisher Scientific). First-strand cDNA synthesis and rapid amplification of cDNA ends (RACE) were performed using the SMARTer™ RACE cDNA amplification kit (Clontech). The primers for RACE were designed based on expressed sequence tags developed from our previous transcriptome library of *A. japonicus* (Sun et al., 2011). Polymerase chain reaction (PCR) amplifications for full-length cDNA confirmation were as follows: 94 °C for 1 min; 30 cycles of 94 °C for 30 s; 50–60 °C for 30 s; 72 °C for 2 min; and 72 °C for 10 min (Eppendorf). PCR products were inserted into the pMD19-T vector (Takara), transformed in JM109 competent cells (Takara) according to the manufacturer's instructions, and five positive clones were selected to confirm *WntA* nucleotide sequence by HUADA (Wuhan, China). The primers for real-time PCR are listed in Table 1. The sequences were analyzed and assembled by DNAStar software to obtain the full-length cDNA sequence.

Table 1
Sequences of RACE PCR and real-time PCR primers.

Primer	Sequence (5′–3′)
RACE-*WntA* F	ACACAAAAGATGGAACTGCCCACTGT
RACE-*WntA* R	ATCCACAATCGAATACATCGCCTGAC
RT-*WntA* F	ACACAAAAGATGGAACTGCCCACTGT
RT-*WntA* R	ATCCACAATCGAATACATCGCCTGAC
NADH F	GTCCTACGACCCAATCTGGA
NADH R	ATGAGCCTTGGTTACGTTGG

2.3. Gene expression analysis by real-time PCR

Total RNA was extracted from residual and new intestinal tissue at different regeneration stages (control, 0, 30 min, 1, 2, 6, 12 h, 3, 7 and 14 days), and DNase-treated using the RNeasy Mini Kit and RNase-Free DNase Set (Qiagen). The quality was determined by agarose gel electrophoresis and quantified using a Nanodrop spectrophotometer (Thermo Fisher Scientific). Subsequently, cDNA templates for real-time PCR were prepared using a Prime Script™ RT Reagent Kit with gDNA Eraser (Takara). The relative expression levels of *WntA* were determined using the SYBR® Premix ExTaq™ (Takara) on an Eppendorf Mastercycler® ep realplex thermocycler (Eppendorf). NADH dehydrogenase was used as an internal control (Sun et al., 2013). The primers for real-time PCR are listed in Table 1. Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method.

2.4. Sequence and phylogenetic analysis

BLAST (Basic Local Alignment Search Tool) was used to analyze the full-length cDNA sequences of *WntA* and predicted amino acid sequences of WntA (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments were conducted using DNAMAN software. The functional sites and domains in the amino acid sequences were predicted by InterProScan (http://www.ebi.ac.uk/interpro/scan.html) and SMART (http://smart.embl-heidelberg.de). Phylogenetic trees using the neighbor-joining method were constructed based on the amino acid sequences using MEGA 5.0 software.

2.5. Protein extraction and western blotting

Total protein extraction and quantitation was performed using T-PER Tissue Protein Extraction Reagent and the BCA protein quantification reagent kit (Thermo Scientific) respectively. Western blot using equal amounts of protein (20 μg/condition) were resolved by 12% polyacrylamide resolving gel electrophoresis (Bio-Rad) and transferred to PVDF membranes by semi-dry transfer (15 V, 15 min). After washing the membranes 2 times with phosphate buffered saline/0.1%Tween-20 (PBST), the membranes were incubated with the corresponding primary