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Expression of *Wnt9*, *TCTP*, and *Bmp1/Tll* in sea cucumber visceral regeneration

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ABSTRACT

We employ non-radioactive in situ hybridization techniques, which combine good tissue morphology preservation with high sensitivity of transcript detection, to map gene expression in the regenerating digestive tube of the sea cucumber *Holothuria glaberrima*. We investigated localization of transcripts of *Wnt9*, *TCTP*, and *Bmp1/Tll*, the genes that have been previously known to be implicated in embryogenesis and cancer. The choice was determined by our long-term goal of trying to understand how the developmental regulatory pathways known to be involved in tumor development can be activated in post-traumatic regeneration without leading to malignant growth. The gene expression data combined with the available morphological information highlight the gut mesothelium (the outer layer of the digestive tube) as a highly dynamic tissue, whose cells undergo remarkable changes in their phenotype and gene expression in response to injury. This reversible transition of the gut mesothelium from a complex specialized tissue to a simple epithelium composed of rapidly proliferating multipotent cells seems to depend on the expression of genes from multiple developmental/cancer-related pathways.

Regeneration, although a distinct phenomenon, is known to rely in part on the mechanisms, that are normally involved in embryogenesis, but recapitulation of the developmental program is never absolute (Carlson, 2007). On the other hand, there are also clear similarities between regeneration and cancer (see, for instance, White and Zon, 2008). The fundamental question here is twofold. First, to what extent does regeneration depend on the mechanisms of normal development? Answering this question will help to understand whether and how re-deployment of developmental programs will help to design new therapeutic approaches to treat the poorly regenerating human body parts. The second issue is what makes regenerative processes stay under control for the benefit of the organism as a whole, without leading to runaway malignant growth. To answer these questions, one needs an extensive knowledge of the mechanisms that underlie regenerative responses.

Arguably, one of the most suitable models in which to seek the answer to the above questions is visceral regeneration in sea cucumbers (holothurians). These marine invertebrates are classified within the phylum Echinodermata, which, on the one hand, is closely related to chordates, and, on the other hand, many members of the phylum are among the best deuterostomian regenerators. Visceral regeneration in sea cucumbers is a naturally occurring phenomenon, which follows autotomy (evisceration) of internal organs in response to adverse stimuli (Byrne, 2001; Hyman, 1955; Wilkie, 2001). A wealth of information has been accumulated on the morphology of the digestive tube in uninjured and regenerating animals and on the cellular mechanisms of regeneration (García-Arrarás et al., 1998; Mashanov and Garcia-Arraras, 2011; Mashanov et al., 2005, 2010). Over the last decade, the molecular events involved in regeneration have also started to be uncovered (Mashanov and Garcia-Arraras, 2011; Mashanov et al., 2010; Ortiz-Pineda et al., 2009; Sun et al., 2011). Of particular interest in the context of the present study is the observation that the regenerating animals showed significant up-regulation of *survivin* and *mortalin*, two genes that are known not only to be involved in embryogenesis and normal stem cell maintenance, but also in cancer progression (Mashanov et al., 2010).

Here, we continue this line of research. A recent microarray study indicated *Wnt9*, *TCTP* and *Bmp1/Tll* as being significantly up-regulated in sea cucumber gut regeneration, as compared with non-injured animals (Ortiz-Pineda et al., 2009). These genes have been previously known to be implicated both in embryogenesis and cancer progression, and here we report their spatio-temporal expression patterns in the regenerating gut of the sea cucumber *Holothuria glaberrima*.

Wnt genes code for highly conserved secreted signaling proteins that are involved in regulation of diverse cell functions, including cell division, cell death, fate decision, cytoskeleton dynamics, cell adhesion, establishing cell polarity, etc. (Croce and McClay, 2008; Nejak-Bowen and Monga, 2011; Taipale and Beachy, 2001; Wend et al., 2010). Wnt9 is one of the least studied members

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of Wnt family. Most published studies have focused on the role of two paralogs, Wnt9a and Wnt9b, in vertebrate embryogenesis. For instance, Wnt9a was found to be essential for normal liver morphogenesis (Matsumoto et al., 2008), and development of iris and corneal epithelium (Fokina and Frolova, 2006). Wnt9b plays an important role in craniofacial morphogenesis (Juriloff et al., 2006; Lan et al., 2006) and also in kidney development (Karner et al., 2011). However, there have been no reports on the role of Wnt9 subfamily members in regeneration.

Translationally controlled tumor protein (TCTP) is a regulator of cell division and cell growth, which is ubiquitously found in all eukaryotes. TCTP is up-regulated in highly proliferating normal tissues and in various cancers. Its expression levels also change significantly under various stress conditions, such as starvation, heat shock, proapoptotic/cytotoxic signals (Bommer and Thiele, 2004; Pollins et al., 2007; Zhu et al., 2008). TCTP is also an important developmental gene. In mice, knockout of TCTP leads to early embryonic lethality associated with defects in cell cycle progression and excessive cell death (Chen et al., 2007a). Its expression is also reported to be associated with the mesoderm development (notochord, presomitic mesoderm and nascent somites) in amphioxus (Chen et al., 2007b). Besides involvement in control of cell proliferation, known cellular functions of TCTP include prevention of apoptosis via inducing degradation of p53, a major tumor suppressor protein (Bommer and Thiele, 2004; Rho et al., 2011). TCTP has also been implicated in regeneration, however, all known reports are restricted to mammals (Jiménez et al., 2005; Pollins et al., 2007; Zhu et al., 2008). Since mammals, and human in particular, have very limited regenerative potential, we therefore, asked whether the role of TCTP in post-traumatic recovery is a more general phenomenon and whether it would be also involved in more robust regeneration responses, such as those that are seen in echinoderms.

Bone morphogenic protein1 (BMP1)/Tolloid (TLD)-like proteins constitute a subgroup of multidomain secreted zinc endopeptidases within the astacin (M12A) family. In mammals, there are four BMP1-like proteinases, including BMP1, Tolloid (TLD), Tolloid-like 1 (TLL1), and Tolloid-like 2 (TLL2). These peptidases play multiple roles in developmental morphogenetic processes. First, they are essential for the proper formation of the extracellular matrix via cleavage of the precursor molecules followed by release of the mature connective tissue proteins (Ge and Greenspan, 2006; Hopkins et al., 2007). The second function of BMP1-like metalloproteinases is activation of TGFβ-like growth factors by liberating them from latent complexes with their inhibitors (Ge and Greenspan, 2006; Hopkins et al., 2007). Among these morphogenetic factors are TGFβ-like BMPs, which are involved in a vast diversity of developmental processes (Bragdon et al., 2011), myostatin and CDF11, negative regulators of muscle growth and neurogenesis, respectively, and TGFβ-1, which controls cell differentiation, growth, and apoptosis (Ge and Greenspan, 2006). Significant up-regulation of *Bmp1* transcription level was reported in human osteosarcoma cells, and can therefore contribute to cancer progression (Lee et al., 1997).

We show here that *Wnt9, TCTP,* and *Bmp1/Tll* transcripts are extensively expressed in sea cucumber gut regeneration and hypothesize that the products of these genes contribute to the enormous injury-triggered plasticity of adult tissues seen in echinoderms.

1. Results

1.1. Sequence analysis

Unlike vertebrates, who have two paralogs Wnt9a and Wnt9b, basal deuterostomes, including echinoderms, are characterized by the presence of a single Wnt9 in their genomes (Supplementary Fig. 1) (Croce and McClay, 2008; Croce et al., 2006). Wnt9 gene of *H. glaberrima*, encodes a 368 amino acid protein, which shares typical characteristics with its vertebrate orthologs (Cox et al., 2010; Fokina and Frolova, 2006; Katoh and Katoh, 2005; Qian et al., 2003) (Supplementary Fig. 1 and 2). As predicted by SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP), it has a 32 amino acid-long N-terminal secretory signal peptide. In eukaryotes, secretory signal peptides allow a protein to be translocated across the endoplasmic reticulum membrane. Once in the endoplasmic reticulum, the proteins are usually targeted for secretion to the outside of the cells, unless they have specific retention signals (Emanuelsson et al., 2007).

The predicted protein sequence of *H. glaberrima* TCTP is 175 amino acids long. It shows a high degree of conservation in the N-terminal 40 amino-acid long region (16 of 40 amino acids in this region are identical between the sea cucumber and human sequences), which is known to be necessary for the anti-apoptotic function of TCTP. Other conserved features include the TCTP1 signature and the C-terminal self-interaction domain (Yang et al., 2005) (Supplementary Fig. 3).

The sequence of the BMP1/TLL protein of *H. glaberrima* has not been reported before. We identified the homolog of Bmp1 among 5173 ESTs representing three cDNA libraries from the normal and regenerating digestive tube (Rojas-Cartagena et al., 2007) by BLAST search against the non-redundant NCBI protein database. The full length of the coding region was reconstructed by performing 5′ and 3′ RACE. Phylogenetic analysis of the sea cucumber BMP1/TLL shows that the sea cucumber protein clusters together with BMP1-like sequences of amphioxus and sea urchin, and that these three proteins form an outgroup relative to the BMP1, TLL1 and TLL2 of vertebrates (Supplementary Fig. 4). Therefore, we prefer to keep the name BMP1/TLL for the sea cucumber sequence. The deduced sea cucumber BMP1/TLD-like protein is 983 amino acids long. It shows exactly the same domain organization as full-length BMP1 of vertebrates (Mac Sweeney et al., 2008) and contains a highly conserved N-terminal metalloprotease domain, two calcium-binding EGF-like domains separated by a CUB domain and flanked on either side by pairs of CUB domains (Fig. 1). The protease domain contains all the residues known to be essential for catalytic activity (Angerer et al., 2006; Mac Sweeney et al., 2008): the three histidine residues that bind the zinc ion in the active center of the enzyme, as well as the conservative glutamic acid and tyrosine (Supplementary Fig. 5). The CUB domains are thought to be involved into protein–protein interactions, whereas the ability of the EGF-like domains to bind calcium ions has been hypothesized to affect the configuration of the BMP1/TLD-like proteinase (Hopkins et al., 2007).

1.2. Introduction to the model

It is necessary here to give the reader a brief background on visceral regeneration in sea cucumbers. Organization of the normal and regenerating digestive tube of the sea cucumber *H. glaberrima* is schematically shown on Fig. 2. In this species, evisceration involves detachment of the entire intestine from the supporting mesentery, as well as from the esophagus and cloaca. The autotomized viscera are discarded through the anus. The early response to injury involves wound healing at the posterior end of the esophageal stump and at the anterior end of the cloaca. Regeneration per se begins with the formation of a solid rudiment in the free edge of the mesentery, which later serves to guide and enclose the developing lumen that forms as two outgrowths from both the anterior and posterior stumps. For the detailed description of the anatomy of the normal and regenerating digestive tube and for a review of the cellular mechanisms involved, the reader is referred to Hyman (1955), García-Arrarás and Greenberg (2001), and Mashanov and Garcia-Arraras (2011).