Research article

Identification and localization of growth factor genes in the sea cucumber, *Holothuria scabra*

Napamanee Kornthong [a,*], Teva Phanaksri [a], Jirawat Saetan [b], Supawadee Duangprom [a], Buranee Lekskul [a], Tipok Vivattanasarn [a], Sineenart Songkoomkrong [a], Prapaporn Jattujan [a], Scott F. Cummins [c,d], Prasert Sobhon [e], Saowaros Suwansa-ard [c,**]

[a] Chulabhorn International College of Medicine, Thammasat University, Rangsit Campus, Pathumthani, 12121, Thailand
[b] Division of Health and Applied Sciences, Faculty of Science, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand
[c] GeneCology Research Centre, University of the Sunshine Coast, Maroochydore, Queensland, 4558, Australia
[d] Faculty of Science, Health, Education and Engineering, University of the Sunshine Coast, Maroochydore, Queensland, 4558, Australia
[e] Department of Anatomy, Faculty of Science, Mahidol University, Rama VI Road, Ratchathewi, Bangkok, 10400, Thailand

ARTICLE INFO

ABSTRACT

The sea cucumber *Holothuria scabra* is both an economically important species in Asian countries and an emerging experimental model for research studies in regeneration and medicinal bioactives. Growth factors and their receptors are known to be key components that guide tissue repair and renewal, yet validation of their presence in *H. scabra* has not been established. We performed a targeted *in silico* search of *H. scabra* transcriptome data to elucidate conserved growth factor family and receptor genes. In total, 42 transcripts were identified, of which 9 were validated by gene cloning and sequencing. The *H. scabra* growth factor genes, such as *bone morphogenetic protein 2A* (*BMP 2A*), *bone morphogenetic protein 5-like* (*BMP5-like*), *neurotrophin* (*NT*) and *fibroblast growth factor 18* (*FGF18*), were selected for further analyses, including phylogenetic comparison and spatial gene expression using RT-PCR and *in situ* hybridization. Expression of all genes investigated were widespread in multiple tissues. However, *BMP 2A*, *BMP5-like* and *NT* were found extensively in the radial nerve cord cells, while *FGF18* was highly expressed in connective tissue layer of the body wall. Our identification and expression analysis of the *H. scabra* growth factor genes provided the molecular information of growth factors in this species which may ultimately complement the research in regenerative medicine.

1. Introduction

Sea cucumbers (Phylum Echinodermata, Class Holothuroidea, Family Holothuriidae) are commercially important species in Asian countries, including China, Hong Kong, Japan, and Indonesia [1, 2]. They have an exceptional ability to quickly regenerate various body parts that are discarded or damaged, and the complexity of their regeneration process is being increasingly studied [3]. Sea cucumbers also produce unique secondary biologically active metabolites [4], which provide nutritional benefits to a consumer or are used as traditional medicine. To date, properties associated with wound healing, neuroprotection, antitumor, anticoagulation, antimicrobial and antioxidant effects have been derived from sea cucumbers extracts [4].

Regeneration can be defined as the regrowth or repair of cells/tissues/organs, which can only be truly found among certain metazoans [5]. Based on what is known, the molecular processes involved in regeneration are remarkably similar to wound healing, which also requires a variety of different tissue and cell lineage changes in forming new tissues [6]. Sea cucumbers are capable of wound healing and regeneration of most tissues and organs, including the body wall, muscle, intestine, respiratory tree, and gonads [7, 8, 9]. For example, *Holothuria glaberrima* undergoes a programmed evisceration process by expelling the digestive tract and associated organs from the body cavity, when it is exposed to noxious stimuli [10, 11]. Several other research studies have investigated the histological changes and cellular events associated with the regeneration of the sea cucumbers [12, 13, 14, 15]. Several genes involved in the regenerative processes of sea cucumbers have been

---

Table 1. Summary of gene-specific primers used for molecular cloning and RT-PCR of *H. scabra* growth factors.

Name of gene	Primer	Direction	Nucleotide sequence	Amplicon size (bp)
BMP2A	BMP2AF	Forward	5′-TACGGAAGACACGACACAAGACAG-3′	299
	BMP2AR1	Reverse	5′-TGGCAGGAAATGGACACTGGCC-3′	
BMP2/4	BMP2/4F	Forward	5′-CGGACGACGGACGGTCGAAGAGGTC-3′	166
	BMP2/4R1	Reverse	5′-GCTTGGTAACCATCCGGCGCCACG-3′	
BMP5-like	BMP5likeF	Forward	5′-CAAGTACAGAATGATCCGATCCCAGGC-3′	298
	BMP5likeR1	Reverse	5′-CCCAACTTAGTGGGGGCGCAACA-3′	
Inhibin	InhibinF	Forward	5′-CCATGGTACTGGGTCGGAGCGTCAG-3′	251
	InhbinR1	Reverse	5′-CCGATTGCCGTAAAGTTGACGGTG-3′	
Myostatin	MSTNF	Forward	5′-GATGGCAGTTCACAGGACGCTC-3′	286
	MSTNR1	Reverse	5′-GGGCGTATTGAAGCATATACTGCCG-3′	
Activin	ActivinF	Forward	5′- CTGCAGGCTTCAGATATAGTAG-3′	326
	ActivinR	Reverse	5′-CGATTTCTCATGGCGCGATT-3′	
TGFbeta2	TGF2F	Forward	5′-CACCGAGAAACAAGCACACCTCGGAG-3′	350
	TGF2R1	Forward	5′- CGGAGAGCAACACGGTGACGGCG-3′	
FGF18	FGFF1	Forward	5′-CCAGCACGTAGCCAGCAACAAATACC-3′	200
	FGFR	Reverse	5′-CTCCTTCCAACGATGTCCCCTC-3′	
Neurotrophin	NTF	Forward	5′-TGGGTGACGGAATGCGATTCAGA-3′	152
	NTR	Reverse	5′-GAAACCCAGTATTCGCCGAGTACA-3′	
H. scabra 16S *mitochondrial* rRNA	16SF	Forward	5′-GAAAGACGAGAAGACCCTGTCGAG-3′	187
	16SR	Reverse	5′-CTTTTTCCGATTACCAGTTTCTGGTTC-3′	

Table 2. Primers used for full-length identification of *H. scabra* growth factors.

Name of transcript	Primer	Direction	Nucleotide sequence
BMP2/4	BMP2/4F3	3RACE	5′-GGAATGACTGGATCGTGGCGCCG-3′
	BMP2/4R5	5RACE	5′-TCGCGGCCTGGGCCTCTCTTCA-3
BMP2A	BMP2AF3	3RACE	5′-CCGCATATTCTGGGCTCGGTGAC-3′
	BMP2AR5	5RACE	5′-CCATTGCCATGAAGCCCTGTACG-3′
BMP5-like	BMP5F3	3RACE	5′-TGTTGCGCCCCCACTAAGTTGGG-3′
	BMP5R5	5RACE	5′-CCAGCAATTCTTCGCTTCCTATCCC-3′
Inhibin	InhibinF3	3RACE	5′-CCTAACATGTTGCCTTGCTGCG-3′
	InhibinR5	5RACE	5′-TGGAGAGACGGTGCTGTGTGG-3′
Myostatin	MSTNF3	3RACE	5′-CCAGATTGTCGATGCAATCGG-3′
	MSTNR5	5RACE	5′-GGTGGATTATCAACAACACTGGC-3′
Activin	ActivinF3	3RACE	5′- GCGCGGTTCACGCGTTAAGAGG-3′
	ActivinR5	5RACE	5′- GGAGAATTCGACCACTGGCGTCG-3′

Table 3. List of genes encoding growth factors and related receptors that were identified or absent in the radial nerve cord and body wall transcriptomes of *H. scabra*. Numbers indicated the number of unique transcripts identified.

Growth factors/Growth factor receptors	Number of hits (Radial nerve cord)	Number of hits (Body wall)
TGF-β2	1	1
TGF-α	0	0
BMPs	3	2
Myostatin/GDF8	1	0
Activin/Inhibin	2	1
NT	1	0
β-NGF	0	0
BDNF	0	0
FGF18	1	1
IGFs	1	0
VEGFs	1	1
EGF	1	1
Ependymin-related	1	0
Univin	0	0
Cryptic	0	0
TGF receptor	4	3
BMP receptor-type II	1	1
Activin receptor	1	1
Tyrosine kinase receptor	3	1
FGF/VEGF receptor	1	1
EGF receptor	1	1
TNF receptor	1	1
Total transcripts	**26**	**16**

Abbreviations: TGF-β, Transforming growth factor-beta; BMP, Bone morphogenetic protein; GDF8, Growth differentiation factor-8; NT, Neurotrophin; NGF, Nerve growth factor; BDNF, Brain-derived neurotrophic factor; FGF, Fibroblast growth factor; VEGF, Vascular endothelial growth factor; EGF, Epidermal growth factor; and TNF, Tumor necrosis factor.

characterized [7, 15, 16, 17], which include genes in the growth factor family, such as myostatin and vascular endothelial growth factor (VEGF) [18, 19, 20, 21]. Moreover, bone morphogenetic proteins (BMPs) are transforming growth factor-beta (TGF-β) superfamily ligands, which have been shown to increase during regeneration in *Apostichopus japonicus* and *H. glaberrima* [20, 21, 22], where they may regulate organ development in echinoderms [20].

Growth factors, in general, are a large and diverse group of polypeptides sharing common properties of stimulating cell growth, proliferation, regulating cell multiplication, differentiation and survival [23, 24, 25]. Their functions range from stimulating cell growth and proliferation, instruction of cell behaviors, guidance the tissue repair and renewal, all of which are essential cellular activities in growth and regeneration. Growth factors can be grouped into families of molecules with related sequences, structures, and consequently, functions. Major growth factor families include platelet-derived growth factor (PDGF), epidermal growth factor (EGF), fibroblast growth factor (FGF), TGF-β, neurotrophin (NT), and insulin-like growth factors (IGFs) [26], the members of which have been well described and studied in their functions in vertebrate models.

Homologs for EGF, FGF, TGF-β, activin, BMP, IGFs, and NT have been identified in invertebrates such as insects, molluscs and echinoderms [27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37]. In *Drosophila melanogaster*, protein members in EGF, FGF and the TGF-β family have been identified and

Figure 1. Occurrence of major growth factors in bilaterians. The occurrence of growth factors in TGF-β superfamily, NT family and FGF8/17/18 family in different taxa of deuterostomes and protostomes. Green and white boxes indicate, respectively, the presence and absence of a given growth factor in a taxonomic group. Asterisks indicate the *H. scabra* growth factors that were identified in this study. Accession numbers of all growth factors presented are provided in **Supplement data 3**.

demonstrated to have important roles in cell survival, morphogenesis, maintenance of primordial germ cells and gametogenesis, as well as embryogenesis [29, 38, 39, 40]. In the mollusc *Aplysia*, a gene encoding a protein similar to human BMP-1 has been identified and proposed to play a role in the regulation of neuronal synaptic connection during long-term sensitization [31]. In echinoderms, the genome of the sea urchin (*Strongylocentrotus purpuratus*) contains most of the genes encoding key proteins in the receptor tyrosine kinase and TGF-β signaling pathways, which suggested that a cytokine signaling pathway in echinoderms is very similar to that of vertebrates [41]. Functionally, these growth factors and related ligands seem to play prominent regulatory roles in the development of sea urchin embryos [41, 42]. However, knowledge of growth factor genes in sea cucumbers is still limited, despite a number of sea cucumber gene resources (i.e., transcriptomes, genomes) are publicly available [43, 44, 45, 46, 47].

Recently, our group explored the proliferative ability of *Holothuria scabra* extracts on mesenchymal stem cells (MSCs) from human placenta [48]. The ability of sea cucumbers in having fast and complete regeneration of their body parts after being injured or damaged indicates that the process of dedifferentiation and redifferentiation are fully retained, in contrast to mammals which may have lost most of these abilities [8, 11, 49, 50]. We postulated that the *H. scabra* extracts might contain growth factors that regulate cell generation in the sea cucumbers. This implication might provide a new treatment for cell and tissue injury in humans, especially when the endogenous pathways might have been lost.

In this study, we performed an *in silico* search of growth factors and their related-receptors in *H. scabra* radial nerve (RN) cord and body wall transcriptomes [51, 52]. Growth factor proteins deduced from *H. scabra* transcriptomes were identified and compared with their homologs in vertebrates, invertebrates, and other echinoderm species, showing their conservation within metazoans. Moreover, some growth factors were selected, and their expression was examined throughout *H. scabra* tissues using reverse transcription polymerase chain reaction (RT-PCR) and *in situ* hybridization.

2. Materials and methods

2.1. Growth factors and related receptors in H. scabra transcriptome: gene mining, sequence alignment, and phylogenetic tree analysis

H. scabra radial nerve and body wall transcriptomes [51, 52] were used for *in silico* search of genes encoding growth factors and their related receptors. A tBLASTn search was conducted using the CLC Main Workbench Version 7.0 (CLC Bio-Qiagen, AsiaPac, Taiwan), with known growth factors proteins derived from vertebrates and invertebrates, as queries. In addition, BLAST search of growth factors in the *H. glaberrima* genome was performed by using tBLASTn program available at *H. glaberrima* genome website (http://ryanlab.whitney.ufl.edu/genomes/Holothuria_glaberrima/) [47]. Search parameters were set as follows: matrix, BLOSUM62; maximum *e*-value, 10. BLAST hit transcripts were retrieved, translated into amino acid sequences, and subsequently used in multiple sequence alignments. Multiple sequence alignment was performed using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and presented with BOXSHADE software (http://sourceforge.net/projects/boxshade/). The amino acid sequence logos were generated by using the Weblogo tool [53]. The phylogenetic tree analysis was conducted using MEGA X software [54] based on the Neighbor-Joining method with bootstrap consensus tree. Bilaterian growth factor proteins used for comparison were collected from the National Center for Biotechnology Information (NCBI) non-redundant protein database.

2.2. Experiment animals and ethics statement

Wild sea cucumbers (*H. scabra*; 200–500 g in body weight with random sex population) were obtained from the Prachuapkirikhan Coastal Fisheries Research and Development Center, Prachuapkirikhan Province, Thailand. There was no requirement for specific permission of sample collections for scientific research purposes. Animals were kept in plastic tanks filled with aerated seawater at ambient temperature for 24 h
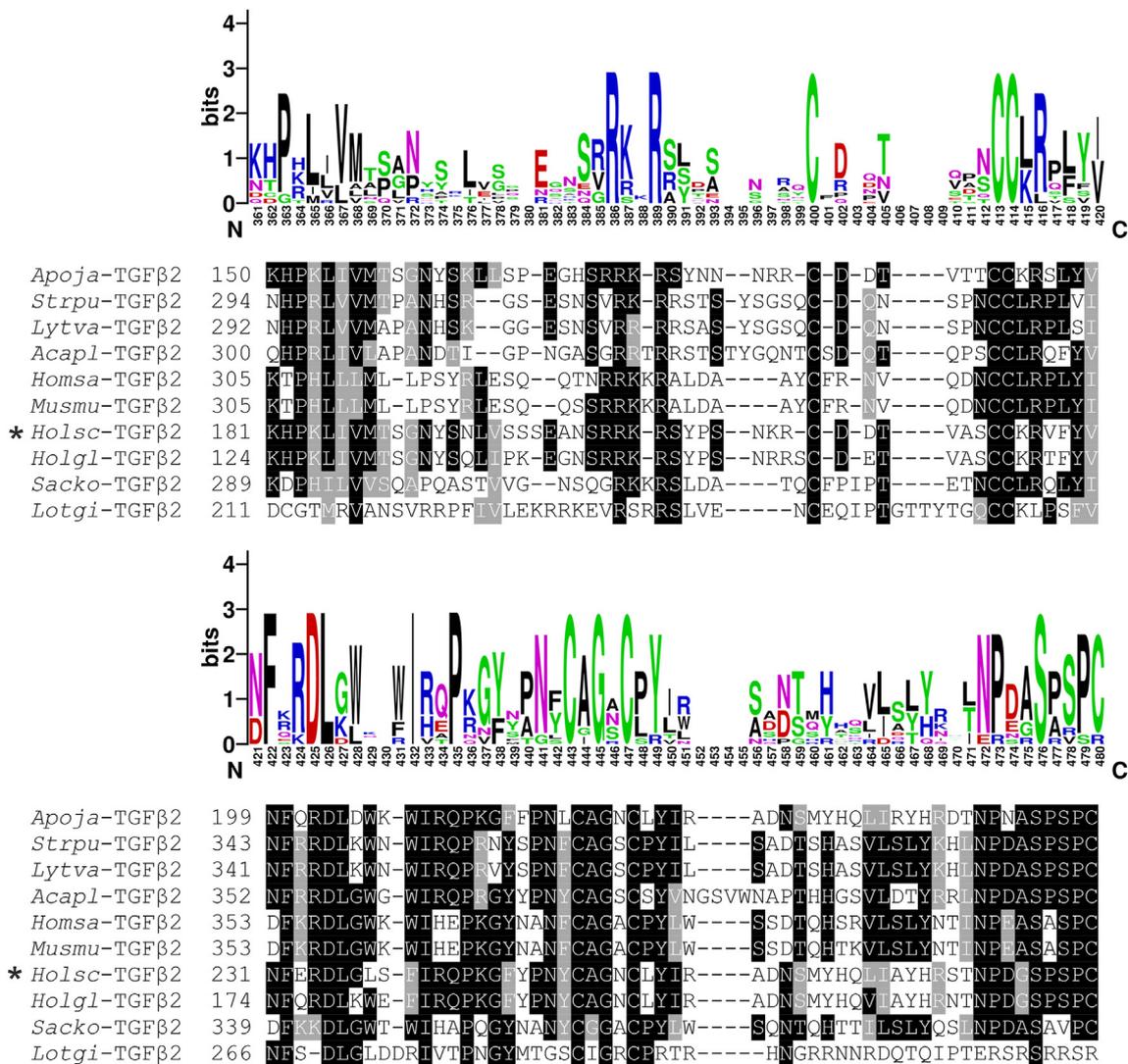
Figure 2. Comparative analysis of the *H. scabra* TGF-β2 protein sequence with other species. The multiple alignment of TGF-β2 of *H. scabra* proteins with TGF-β2 proteins in other species from chordates, hemichordates, molluscs, and other echinoderms. Black shading in the multiple alignment indicates conserved amino acids while grey shading indicates similar amino acids. The asterisks indicate *H. scabra* TGF-β2 found in this study. The sequence logo above the alignment shows the conservation of amino acid residues. Species abbreviations: *Homsa, Homo sapiens; Musmu, Mus musculus; Sacko, Saccoglossus kowalevskii; Lotgi, Lottia gigantea; Apoja, Apostichopus japonicus; Lytva, Lytechinus variegatus; Strpu, Strongylocentrotus purpuratus; Acapl. Acanthaster planci; Holgl, Holothuria glaberrima; Holsc, Holothuria scabra.* Accession numbers of TGF-β2 protein sequences presented are provided in **Supplement data 4**.

before being sacrificed. Before tissue collection, sea cucumbers were anesthetized for 5–10 min in 20 L seawater containing 0.125 mL/L of clove oil, after which various tissues were collected and either immediately frozen in liquid nitrogen or fixed in 4% paraformaldehyde. Frozen tissues were stored at -80 °C until use for RNA extraction, while fixed tissues were further processed for histology preparation and subsequent *in situ* hybridization. The experiments were performed according to the guidelines on the care and use of animals for scientific purposes provided by the Institutional Care and Use Committee of Thammasat University. This study was specifically approved by the Animal Care and Use Committee of Thammasat University, National Research Council of Thailand (NRCT), Protocol Number 019/2561. All efforts were made to minimize the suffering of animals.

2.3. RNA isolation and full-length sequence identification of growth factors

Total RNAs from the RN cord and inner part of the body wall (IW) were prepared from each tissue using Tripure isolation reagent (Roche, Germany), following the manufacturer's protocol, and kept at -80 °C until use. The purity and quantity of each RNA sample were measured by a spectrophotometer at 260 and 280 nm. Complementary DNA (cDNA) was subsequently prepared using QuantiNova Reverse Transcription Kit (Qiagen, Germany), and used as a template for gene amplification by PCR. Details of gene-specific forward and reverse primers used in PCR were included in Table 1. Thermocycling conditions used for PCR amplification were set as follows: 1 cycle at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 45 s at 55–62 °C, and 45 s at 72 °C, with a final extension of 10 min at 72 °C. To identify the full-length sequences of selected genes, 3′ RACE and 5′ RACE were performed. cDNA libraries from RN and IW were constructed using the SMART™ cDNA library construction kit (Clontech, CA, USA), following the manufacturer's protocols. Universal primers (provided in the kit) and gene-specific primers were used to obtain a complete gene sequence (Table 2). Thermocycling conditions were: 1 cycle at 94 °C for 5 min, followed by 35 cycles of 30 s at 94 °C, 30 s at 55–60 °C, and 1 min at 72 °C, with a final extension of 10 min at 72 °C. All amplification products were analyzed using 2% agarose gel electrophoresis. The amplicon was purified using a GeneJET gel extraction kit (Thermo Scientific, USA), and cloned into a pGEM®-T Easy vector (Promega, USA). Plasmids with insert sequences were purified using a GeneJET Plasmid Mini-prep Kit (Thermo Scientific, USA), and
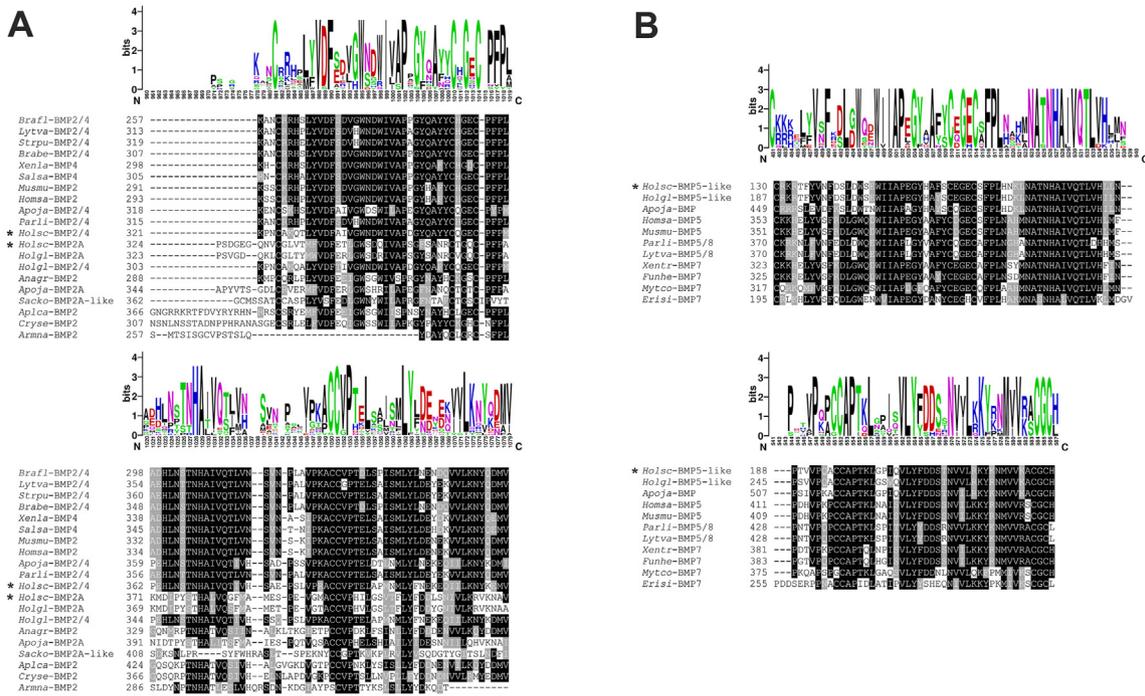
Figure 3. Comparative analysis of the *H. scabra* BMP2A, BMP2/4 and BMP5-like protein sequences with other species. **(A)** Comparative analysis of the *H. scabra* BMP2A and BMP2/4 protein sequences with other species. The multiple alignment of BMP2A and BMP2/4 of *H. scabra* proteins with BMP2, BMP4, BMP2/4 and BMP2A proteins in other species from chordates, hemichordates, molluscs, insects, crustaceans, and echinoderms. **(B)** Comparative analysis of the *H. scabra* BMP5-like protein sequences with other species. The multiple alignment of BMP5-like of *H. scabra* proteins with BMP, BMP5, BMP5/8 and BMP7 proteins in other species from chordates, molluscs, crustaceans, and echinoderms. Black shading in the multiple alignment indicates conserved amino acids while grey shading indicates similar amino acids. The asterisks indicate *H. scabra* BMP2A BMP2/4 and BMP5-like that were found in this study. The sequence logo above the alignment shows the conservation of amino acid residues. Species abbreviations: *Homsa, Homo sapiens; Musmu, Mus musculus; Xenla, Xenopus laevis; Salsa, Salmo salar; Anagr, Anabarilius graham; Brabe, Branchiostoma belcheri; Brafl, Branchiostoma floridae; Aplca, Aplysia californica; Cryse, Cryptotermes secundus; Armna, Armadillidium nasatum; Sacko, Saccoglossus kowalevskii;* Cryse, *Cryptotermes secundus; Lytva, Lytechinus variegatus; Strpu, Strongylocentrotus purpuratus; Parli, Paracentrotus lividus; Apoja, Apostichopus japonicus; Funhe, Fundulus heteroclitus; Xentr, Xenopus tropicalis; Mytco, Mytilus coruscus; Erisi, Eriocheir sinensis; Holgl, Holothuria glaberrima; Holsc, Holothuria scabra.* Accession numbers of BMP protein sequences presented are provided in **Supplement data 5**.

then sequenced by Macrogen (Macrogen Inc., Korea). The sequence identification was conducted according to the previously described protocol [55, 56].

2.4. Gene tissue distribution by reverse transcription-polymerase chain reaction (RT-PCR)

To investigate *H. scabra* growth factor expression, RNA was extracted from various tissues of five *H. scabra* individuals, including radial nerve cord (RN), nerve ring (NR), inner part of the body wall (IW), testis (TT), ovary (OV), respiratory tree (RT), intestine (IN) and longitudinal muscle (LM), using Tripure isolation reagent (Roche, Germany). The purity and quantity of each RNA sample were measured by a spectrophotometer at 260 and 280 nm cDNA was subsequently prepared using QuantiNova Reverse Transcription Kit (Qiagen, Germany), and cDNA used as a template for PCR using gene-specific primers of the *H. scabra BMP2A, BMP2/ 4, BMP5-like, TGF-β2, activin, inhibin, myostatin, NT,* and *FGF18* genes (Table 1). The RT-PCR was determined within 30 cycles to allow the comparison of amplicons developed from an internal control (*H. scabra 16S mitochondrial rRNA* gene, GenBank accession no: JQ657264.1). No addition of template (i.e., reactions without cDNA) was used as negative controls.

2.5. Light microscopy and hematoxylin and eosin staining

H. scabra fixed tissues were dehydrated in a series of increasing concentrations of ethanol (70, 80, 90, and 100%), transferred to toluene, and infiltrated and embedded in paraffin. Sections of the embedded tissues were cut at 6 μm thickness using a rotary microtome (Leica RM2235,

Germany). They were then placed onto slides coated with the 3-aminopropyl triethoxy-silane solution (Sigma Co., St. Louis, MO). The hematoxylin and eosin (H&E) method was performed following the previously described protocol [57]. The sections were deparaffinized with xylene and then rehydrated in a graded series of ethyl alcohol (100, 95, 90, 80, and 70%), for 5 min each. They were stained with hematoxylin and eosin, mounted with Permount (Bio-optica, Milan, Italy), and then examined and photographed a Leica compound microscope equipped with a digital camera (Leica, Germany).

2.6. Gene tissue distribution by in situ hybridization

In situ hybridization was conducted following the previously described protocol [56]. Recombinant plasmids containing *H. scabra* growth factor genes (*H. scabra BMP2A, H. scabra BMP5-like, H. scabra NT,* and *H. scabra FGF18*) were used as templates for PCR amplification, using M13 forward (5′-GTAAAACGACGGCCAGT-3′) and M13 reverse primers (5′-AACAGCTATGACCATG-3′). PCR products were analyzed on agarose gel electrophoresis and subsequently extracted using GeneJET Gel Extraction Kit (Thermo Scientific, USA). Approximately 250 ng of linearized DNA was used as the template to produce DIG-labeled sense-strand and anti-sense RNA riboprobes (DIG-RNA labeling kit SP6/T7, Roche, Germany).

H. scabra RN with the inner part of body wall was dissected out and fixed with 4% paraformaldehyde for 12–15 h. The tissues were then processed through routine tissue processing before being embedded in paraffin blocks. Consecutive paraffin sections of 5–6 μm thick were cut and mounted onto silane-coated slides. The tissue sections were dewaxed in fresh xylene (twice for 10 min each) and
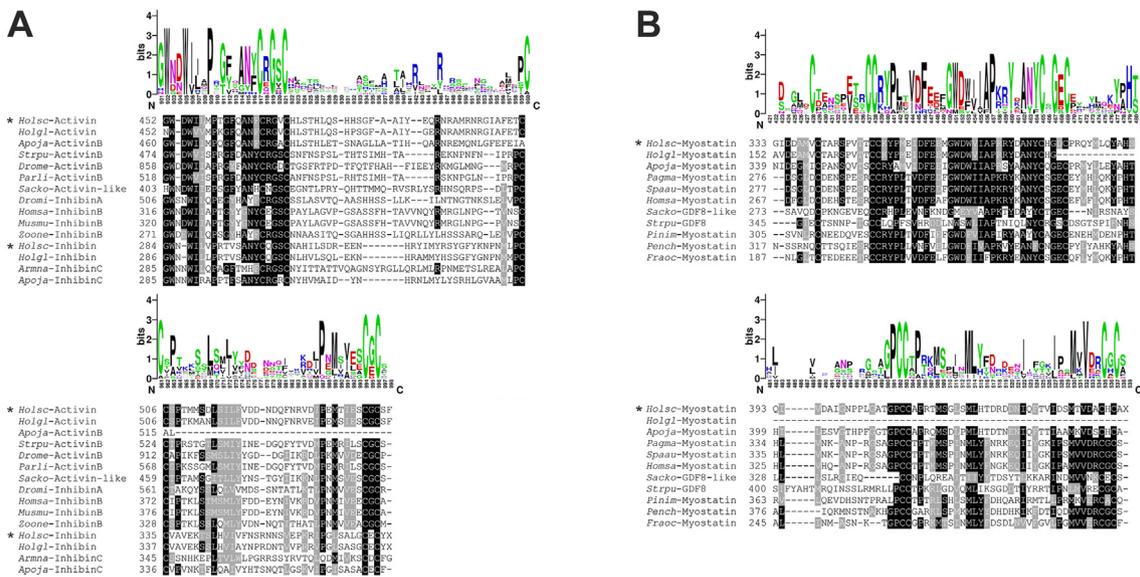
Figure 4. Comparative analysis of the *H. scabra* activin/inhibin and myostatin protein sequences with other species. **(A)** Comparative analysis of the *H. scabra* activin and inhibin protein sequences with other species. The multiple alignment of activin and inhibin of *H. scabra* proteins with activin and inhibin proteins in other species from chordates, hemichordates, molluscs, insects, crustaceans, and echinoderms. **(B)** Comparative analysis of the *H. scabra* myostatin protein sequence with other species. The multiple alignment of myostatin of *H. scabra* proteins with myostatin proteins in other species from chordates, hemichordates, molluscs, insects, crustaceans, and echinoderms. Black shading in the multiple alignment indicates conserved amino acids while grey shading indicates similar amino acids. The asterisks indicate *H. scabra* activin, inhibin and myostatin that were found in this study. The sequence logo above the alignment shows the conservation of amino acid residues. Species abbreviations: *Homsa, Homo sapiens; Musmu, Mus musculus; Sacko, Saccoglossus kowalevski; Drome, Drosophila melanogaster; Dromi, Drosophila miranda; Armna, Armadillidium nasatum; Zoone, Zootermopsis nevadensis; Parli, Paracentrotus lividus; Apoja, Apostichopus japonicus; Saau, Sparus aurata; Pench, Penaeus chinensis; Feaoc, Frankliniella occidentalis; Pinim, Pinctada imbricata; Strpu, Strongylocentrotus purpuratus; Holgl, H. glaberrima; Holsc, Holothuria scabra*. Accession numbers of activin, inhibin and myostatin protein sequences presented are provided in Supplement data 6.

rehydrated with a decreasing concentration of ethanol solutions. The sections were treated with a TE buffer (100 mM Tris-HCl, 50 mM EDTA, pH 8.0) containing RNAse-free proteinase K (5 µg/ml) (Roche, Germany), at 37 °C for 30 min. Post-fixation was performed with diethyl pyrocarbonate (DEPC)-treated PBS containing 4% paraformaldehyde, at 4 °C for 5 min. The sections were then incubated in prehybridization buffer [4x sodium saline citrate (SSC) containing 50% deionized formamide], at 37 °C for 10 min. After prehybridization, the buffer was removed, and each section overlaid with 200 µl of hybridization buffer (40% deionized formamide, 10% dextran sulfate, 1xDenhardt's solution, 4xSSC, 10 mM DTT, 1 mg/ml yeast t-RNA, 1 mg/ml denatured and sheared salmon sperm DNA) containing 10 ng of either sense or anti-sense DIG-labeled RNA probe. Sections were incubated with hybridization buffer containing probes at 42 °C for overnight within a humid chamber. Sections were then washed twice in 2xSSC and 1xSSC, respectively (15 min each) on a shaking platform at 37 °C before being incubated in 20 µg/ml RNase A-containing TNE buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) for 30 min at 37 °C. After incubation, sections were washed twice (10 min each) in buffer 1 [100 mM Tris-HCl (pH 7.5), 150 mM NaCl], incubated with blocking solution [buffer 1 containing 0.1% Triton X-100 and 2% normal sheep serum (Sigma)] for 30 min. Detection of DIG-labeled probes was performed by incubating sections in buffer 1 containing sheep anti-DIG-alkaline phosphatase (Roche, Germany) at dilution of 1:500 for 12–16 h. After being washed twice (10 min each) in buffer 1 and buffer 2 [100 mM Tris-HCl (pH 9.5), 100 mM NaCl, 50 mM MgCl₂], respectively, sections were incubated with buffer 2 containing NBT and BCIP (Roche, Germany) in the dark for approximately 1–2 h. At the end of the color development, sections were washed briefly in DEPC-treated H₂O, before being mounted in 90% glycerol. Finally, sections were observed and photographed using a Leica compound microscope equipped with a digital camera (Leica, Germany).

3. Results

3.1. Identification of growth factors and related receptors in the H. scabra radial nerve and body wall transcriptomes

An *in silico* search of growth factor family genes and related receptors in *H. scabra* transcriptomes, revealed 26 transcripts from the radial nerve cord and 16 transcripts from the body wall with high confidence (Table 3 and Supplement Data 1). All growth factor transcripts were submitted and available in the NCBI database with accession numbers MW728942 to MW728950 (Supplement Data 2). These were categorized into 3 major growth factor families based on existing knowledge about the effect of growth factors, which included TGF-β, NT, and FGF families. The existence of growth factors in *H. scabra* and other bilaterians was compared to provide a summary of evolutionary conservation of major growth factors within TGF-β, NT and FGF8/17/18 families (Figure 1 and Supplement Data 2 and 3). The members of the growth factors in the TGF-β, NT and FGF8/17/18 families were highly conserved among vertebrates and invertebrates. At least 1 form of the TGF-β gene was present in the representative species of a given taxon, except in the annelids where other members of the TGF-β superfamily such as myostatin/GDF8, activin B, and BMP proteins were only found. Within the NT superfamily, NT was conserved among Bilaterians, while NGF and BDNF were present only in a few groups of phyla. Lastly, proteins in FGF8/17/18 families were present in almost all taxa investigated, except in Annelida. Meanwhile, the high conservation in amino acid composition within functional domains of TGF-β, NT, and FGF8/17/18 family members in humans and sea cucumbers was also presented (Supplement Data 2).

3.1.1. TGF-β superfamily

The majority of *H. scabra* growth factor transcripts identified in this study belonged to the TGF-β superfamily, including *TGF-β2, BMP2A, BMP2/4, BMP5-like, activin, inhibin* and *myostatin* (Table 3). Full-length transcripts were obtained for all, besides *TGF-β2* for which a partial-
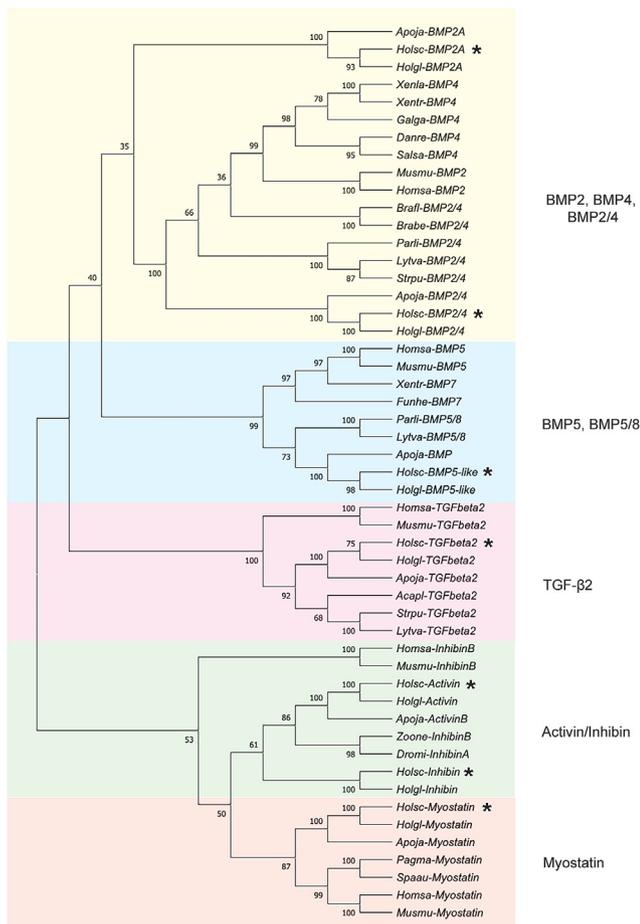
Figure 5. Phylogenetic tree analysis of TGF-β superfamily proteins from *H. scabra* and other species from different phyla. The phylogenetic tree was constructed based on the Neighbor-Joining method (number of amino acid substitution model). The number at the nodes represent bootstrap values at an approximate level of percent confidence from 1000 bootstrap replicates. The asterisks indicate *H. scabra* TGF-β2, myostatin, inhibin, activin, BMP2, BMP2/4 and BMP5-like that were found in this study.

length transcript was elucidated (Supplement Data 1–2). The deduced *H. scabra* growth factor proteins within the TGF-β superfamily were compared with homologs from other species. Conserved amino acids were observed throughout the entire length of the protein, especially the active peptide region.

Based on full-length sequence alignment, *H. scabra* TGF-β2 shared 28–30 % similarity to their homologous proteins in chordates and hemichordates such as *Homo sapiens* TGF-β2, *Mus musculus* TGF-β2 and *Saccoglossus kowalevskii* TGF-β2, and 21% similarity to *Lottia gigantea* (a representative mollusc) TGF-β2, while *H. scabra* TGF-β2 shared 37–66% similarity to other echinoderms TGF-β2, including *A. japonicus* TGF-β2, *S. purpuratus* TGF-β2 and *Acanthaster planci* TGF-β2. *H. scabra* TGF-β2 shared 80% similarity to *H. glaberrima* TGF-β2 (Figure 2, Supplement Data 4). The *H. scabra* BMP2A and BMP2/4 proteins were grouped and compared to other BMP2, BMP4 and BMP2/4 in other species from chordates, hemichordates, molluscs, insects, crustaceans, and other echinoderms. *H. scabra* BMP2/4 protein showed 50–92% similarity with BMP2/4 of other echinoderm species, 42–50% similarity to chordates BMP 2 and BMP 4, cephalochordates and hemichordates BMP2/4, and 27–32% similarity to BMP2s from molluscan, insect and crustacean representative species (Figure 3A, Supplement Data 5). *H. scabra* BMP2A protein showed 83% similarity with *H. glaberrima* BMP2A, 38% similarity with *A. japonicus* BMP2A, and 20–34% similarity with BMP2/BMP4 in representative species from chordates, hemichordates, molluscs, insects,

and crustaceans (Figure 3A, Supplement Data 5). The *H. scabra* BMP5-like displayed 71–90% similarity with *A. japonicus* BMP and *H. glaberrima* BMP5-like. While *H. scabra* BMP5-like shared 49–50% with other echinoderms BMP5/8, and 36–46% similarity to chordates BMP5, molluscs BMP7, and crustaceans BMP7 (Figure 3B, Supplement Data 5).

According to the highly complex relationship of activin and inhibin, the *H. scabra* activin and inhibin proteins were grouped and compared to other activin and inhibin of other species. Based on protein precursor sequences, *H. scabra* activin shared 84% similarity with *H. glaberrima* activin and 50% similarity to *A. japonicus* activin B. It shared 25–27% similarity with chordates, hemichordates, insects, and other echinoderms activin. While *H. scabra* inhibin showed 71% similarity with *H. glaberrima* inhibin and 42% similarity to *A. japonicus* inhibin C, it also displayed 20–24% similarity with chordates, insects, and crustaceans inhibin (Figure 4A, Supplement Data 6). The *H. scabra* myostatin, also referred to as growth differentiation factor-8 (GDF-8) was compared to myostatin or GDF-8 in other species. *H. scabra* myostatin shared 90% similarity with *H. glaberrima* myostatin, 54% similarity to *A. japonicus* myostatin and 20–29 % similarity to their homologous myostatin and GDF-8 in chordates, hemichordates, insects, molluscs, crustaceans, and echinoderms (Figure 4B, Supplement Data 6).

A phylogenetic analysis was constructed based on *H. scabra* TGF-β superfamily protein sequences and examples of random and diversity within the TGF-β superfamily. It indicated that *H. scabra* TGF-β2, BMP2A, BMP2/4, BMP5-like, activin, myostatin and inhibin were closely related to other homolog proteins in other species and phyla, such as chordates, hemichordates, molluscs, insects, and echinoderms (Figure 5). In addition, the phylogenetic tree displayed five distinct branches: (1) BMP2A/ BMP2/4, (2) BMP5/BMP5/8, (3) TGF-β2, (4) myostatin, and (5) activin/ inhibin.

3.1.2. Neurotrophin (NT)

A sequence of *H. scabra* NT was predicted and subsequently compared with nerve growth factors (NGFs), brain-derived neurotrophic factor (BDNFs), and NTs from other species and phyla. Conservation as shown by multiple sequence alignment of protein precursors confirmed their placement in the same family (Figure 6A and Supplement Data 7). *H. scabra* NT shared 28–30% similarity to *H. glaberrima* NT and *S. purpuratus* NT5, and 20–23% similarity to NTs of other chordates NTs, hemichordate NGF-like. Moreover, *H. scabra* NT shared 19–20% similarity to chordates NGF and BDNFs, crustaceans NT and molluscs NT. All NTs displayed spatial conservation of 6 cysteine residues (Figure 6A). Phylogenetic tree analysis using NT family proteins showed that *H. scabra* NT was most closely related to *S. purpuratus* NT5 and molluscan NT1-like (Figure 6B). Moreover, *H. scabra* NT was rooted with chordate NGF, BDNF, and NT3 clusters.

3.1.3. Fibroblast growth factor 18 (FGF18)

A sequence of FGF18 in *H. scabra* was identified with high confidence to other echinoderm FGF18. The *H. scabra* FGF18 deduced precursor protein showed the highest similarity to *H. glaberrima* FGF18 (90%). *H. scabra* FGF18 shared 36–39% to those of the starfish, *Asterias rubens* and *Acanthaster planci* FGF18, and 29% similarity to the lancelets, *Branchiostoma floridae* and *Branchiostoma lanceolatum* FGF8/17/18 proteins. *H. scabra* FGF18 shared 27% similarity to molluscs, *Mizuhopecten yessoensis* FGF18, and 19–30% similarity to chordates FGFs and other echinoderms FGFs (Figure 7A and Supplement Data 8). Phylogenetic tree analysis showed that *H. scabra* FGF18 was most closely related to FGF18s in other sea cucumber and sea stars. Sea cucumber FGFs were also rooted with the chordates FGF8, FGF17, FGF18, and lancelets (cephalochordate representatives) FGF8/17/18 cluster (Figure 7B).

3.1.4. Growth factor-related receptors

We identified 12 growth factor-related receptors in *H. scabra* radial nerve cord transcriptomes, of which 9 were also present in body wall transcriptomes, including tyrosine kinase receptor, TGF-β receptor, BMP
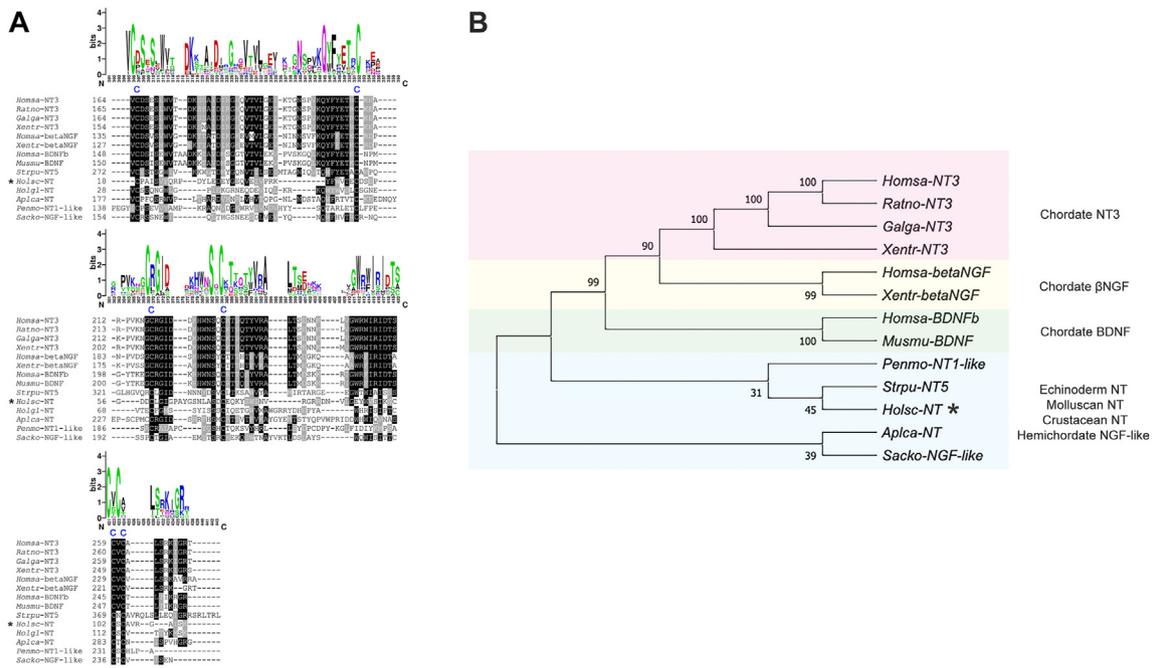
Figure 6. Comparative analysis of the *H. scabra* neurotrophin (NT) with other species. (**A**) The alignment of *H. scabra* NT protein with the ligands of NT family, including NGF, BDNF, and NT from other species of different phyla. Black shading in the multiple alignment indicates conserved amino acids while grey shading indicates similar amino acids. The blue letter C's indicate the conserved cysteine residues in the multiple sequence alignment. The sequence logo above the alignment shows the conservation of amino acid residues. (**B**) Phylogenetic tree analysis of *H. scabra* NT and the ligands of NT family of other species from different phyla. The phylogenetic tree was constructed based on the Neighbor-Joining method (number of amino acid substitution model). The number at the nodes represent bootstrap values at an approximate level of percent confidence from 1000 bootstrap replicates. The asterisks indicate *H. scabra* NT found in this study. Species abbreviations: *Homsa, Homo sapiens; Musmu, Mus musculus*; *Ratno, Rattus norvegicus*; *Galga, Gallus gallus*; *Xentr, Xenopus tropicalis*; *Sacko, Saccoglossus kowalevskii*; *Aplca, Aplysia californica*; *Penmo, Penaeus monodon*; *Strpu, Strongylocentrotus purpuratus*; *Holgl, Holothuria glaberrima*; *Holsc, Holothuria scabra*. Accession numbers of NGF, BDNF, and NT protein sequences presented are provided in Supplement data 7.
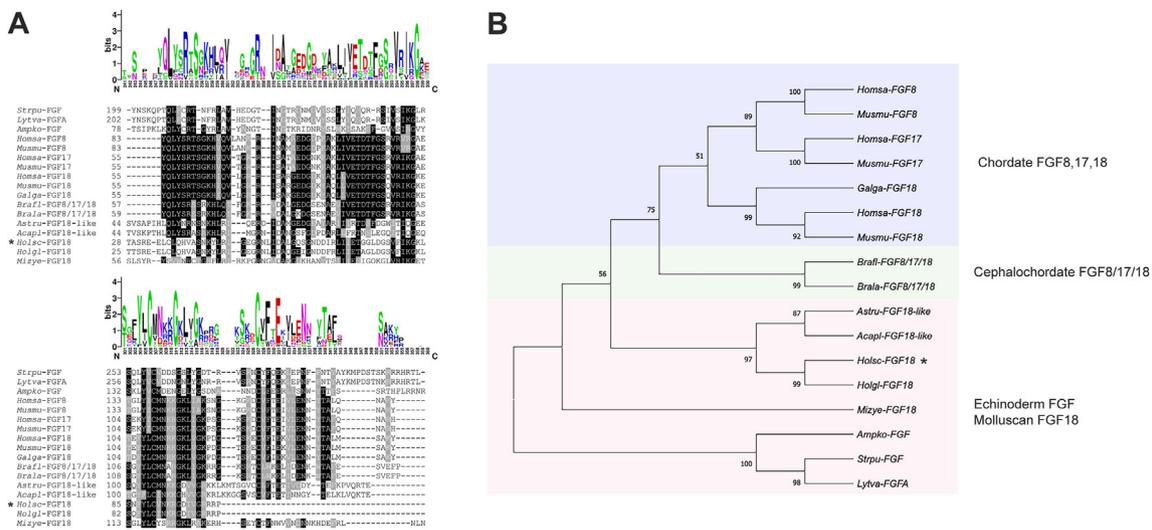

Figure 7. Comparative analysis of *H. scabra* fibroblast growth factor 18 (FGF18). (**A**) The alignment of *H. scabra* FGF protein with the ligands of FGF family, including FGF, FGF8, FGF17, FGF18, FGF8/17/18 and FGF18-like from other species of different phyla. Black shading in the multiple alignment indicates conserved amino acids while grey shading indicates similar amino acids. The sequence logo above the alignment shows the conservation of amino acid residues. (**B**) Phylogenetic tree analysis of *H. scabra* FGF18 and the ligands of FGF family of other species from different phyla. The phylogenetic tree was constructed based on the Neighbor-Joining method (number of amino acid substitution model). The number at the nodes represent bootstrap values at an approximate level of percent confidence from 1000 bootstrap replicates. The asterisks indicate *H. scabra* FGF18. Species abbreviations: *Homsa, Homo sapiens; Musmu, Mus musculus; Galga, Gallus gallus; Brala, Branchiostoma lanceolatum; Brafl, Branchiostoma floridae; Lytva, Lytechinus variegatus; Ampho, Amphipholis kochii; Mizye, Mizuhopecten yessoensis; Strpu, Strongylocentrotus purpuratus; Astru, Asterias rubens; Acapl, Acanthaster planci; Holgl, Holothuria glaberrima; Holsc, Holothuria scabra*. Accession numbers of FGF protein sequences presented are provided in Supplement data 8.
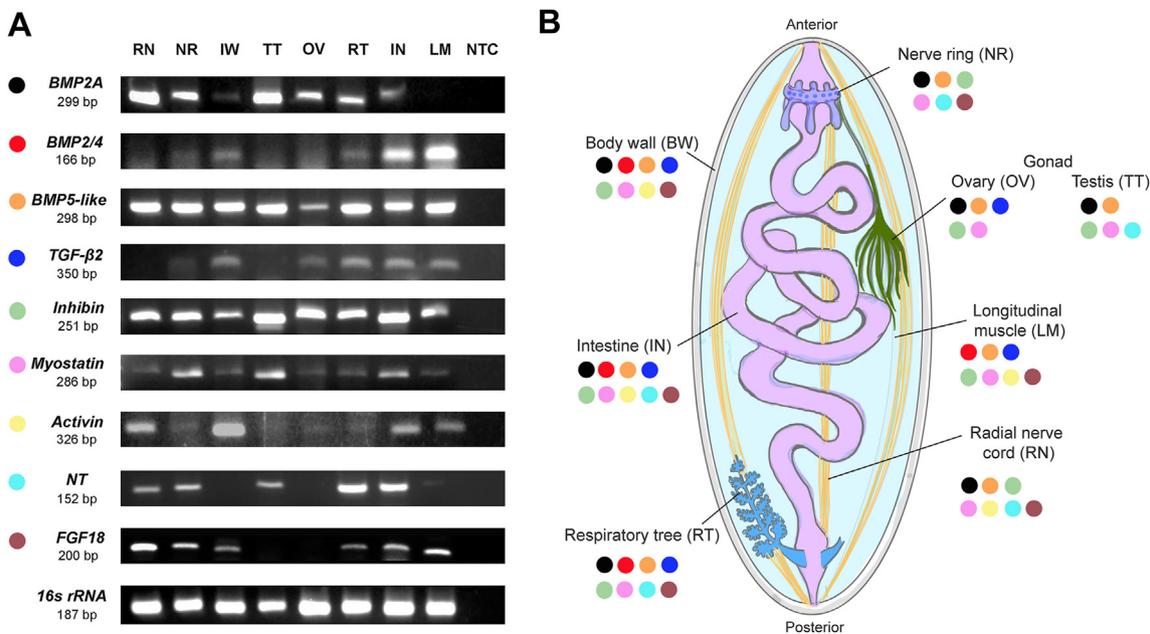
Figure 8. (A) Tissue-specific expression of *H. scabra BMP2A, BMP2/4, BMP5-like, myostatin, inhibin, activin, TGF-β2, NT*, and *FGF18* mRNA using RT-PCR (amplicon sizes about 150–350 bp). The *16s rRNA* expression was used as a positive control. The non-adjusted agarose gel images of *H. scabra* growth factors and *16s rRNA* RT-PCR were provided in Supplement data 9. (B) Schematic diagram of *H. scabra* anatomy and the presence of various growth factors in each organ. The color code of circles in (B) represents different growth factors, which corresponds to those in (A). Abbreviations: radial nerve cord (RN), nerve ring (NR), inner part of the body wall (IW), testis (TT), ovary (OV), respiratory tree (RT), intestine (IN) and longitudinal muscle (LM). Negative controls (no template control; NTC).

receptor-type II, activin receptor, FGF/VEGF receptor, EGF receptor and TNF receptor (Table 3 and Supplement Data 1). The presence of growth factor-related receptors was predicted based on our identification of *H. scabra* growth factors and previously existing knowledge about the growth factor-related receptor binding function. The most abundant receptor transcripts in *H. scabra* transcriptomes were tyrosine kinase receptor and TGF receptor. Three transcripts, including *TNF receptor, TGF-β receptor* and *FGF/VEGF receptor*, were identified as full-length, while others were partial-length (Supplement Data 1).

3.2. Tissue expression of H. scabra growth factors using RT-PCR and in situ hybridization

The presence of *H. scabra BMP2A, BMP2/4, BMP5-like, TGF-β2, activin, inhibin* and *myostatin, NT* and *FGF18* in different *H. scabra* tissues was examined by RT-PCR (Figure 8A and Supplement Data 9). Gene expression for all growth factors was widespread across the tissues analyzed (Figure 8A, B). *H. scabra BMP2A* transcript was found in the RN cord, NR, IW, TT, OV, RT and IN, while *H. scabra BMP2A* was not detected in the LM. *H. scabra BMP5-like* and *H. scabra Inhibin* were detected in all examined tissues, whereas LM. *H. scabra BMP2/4* was detected in the IW, RT, IN and LM. While *H. scabra TGF-β2* was expressed in all tissues with different level of expression, except in the RN cord, NR and TT. *H. scabra Myostatin* was also detected in all tissues with different level of expression. *H. scabra Activin* was expressed in the RN cord, IW, IN and LM. *H. scabra NT* was detected in the RN cord, NR, TT, RT and IN. Lastly, *H. scabra FGF18* was detected in all tissues, except in the gonads (TT and OV). No amplification was detected in the negative controls.

In situ hybridizations were performed for *H. scabra BMP 2A, BMP5-like, NT* and *FGF18* in *H. scabra* RN cord and other structures of the IW. First, histological staining (with H&E) showed the anatomical structures that constitute the RN cord and associated inner body wall, including the LM band, water vascular canal, hemal lacuna and connective tissue (CT) layer (Figure 9A). Higher magnification of the RN cord showed two parallel bands of nerve tissue, a thick ectoneural neuroepithelium (EN) and relatively thin hyponeural neuroepithelium (HN), separated by CT (Figure 9B). The epineural canal was located at the outer region of the

EN, whereas the hyponeural canal was situated most inner to the HN. As a representative negative control, a sense-strand riboprobe of *H. scabra BMP 2A* was used to show no positive signals within *H. scabra* tissues (Figure 9C). The sense-strand riboprobe of *H. scabra BMP5-like, NT* and *FGF18* detection also performed, showing no positive signals (data not shown). All other analysis was performed with anti-sense strand riboprobes of *H. scabra BMP2A, BMP5-like, NT* and *FGF18*. *H. scabra BMP2A* was detected in RN cord cells present in the EN, as well as the thin CT between EN and HN of the RN cord, and the CT layer of the body wall (Figure 9D–F). *H. scabra BMP5-like* was localized to cells of the EN and, at less intensity, in the thin CT between EN and HN of the RN cord (Figure 9G, H). Moreover, *H. scabra BMP5-like* was detected in the cells of the NR and hemal lacuna (Figure 9G, I). *NT* was detected in cells of the EN, as well as the thin CT between EN and HN of the RN cord, the CT layer and the hemal lacuna (Figure 9J–L). *FGF18* was found at less intensity in the RN cord and with stronger intensely in the hemal lacuna and the CT layer (Figure 9M–O). In addition, *H. scabra BMP 2A, BMP5-like, NT*, and *FGF18* were detected in the LM band at a lower intensity (Figure 9D–O).

4. Discussion

Growth factors are considered to be involved in many processes throughout metazoans including development, homeostasis, and immunity [58]. Experimental evidence has indicated that many growth factors found in vertebrates are phylogenetically ancient [59]. Therefore, their identification in Holothuroidea, as described in our study, provides insight into their possible roles in growth and regeneration processes. Specifically, we describe the identification and distribution of the *H. Scabra* TGF-β superfamily, NT, and FGF.

Seven proteins belonging to the TGF-β superfamily were identified from *H. scabra*, including *TGF-β2, BMP2A, BMP2/4, BMP5-like, activin, inhibin* and *myostatin*. To date in the echinoderms, TGF-β superfamily genes have been reported in Holothuridae, Asteroidea and Echinoidea [14, 21, 41, 43, 60, 61]. Our observation was that various TGF-β superfamily members were commonly presented in deuterostome lineages. Based on RT-PCR, expression of *H. scabra* TGF-β superfamily genes was
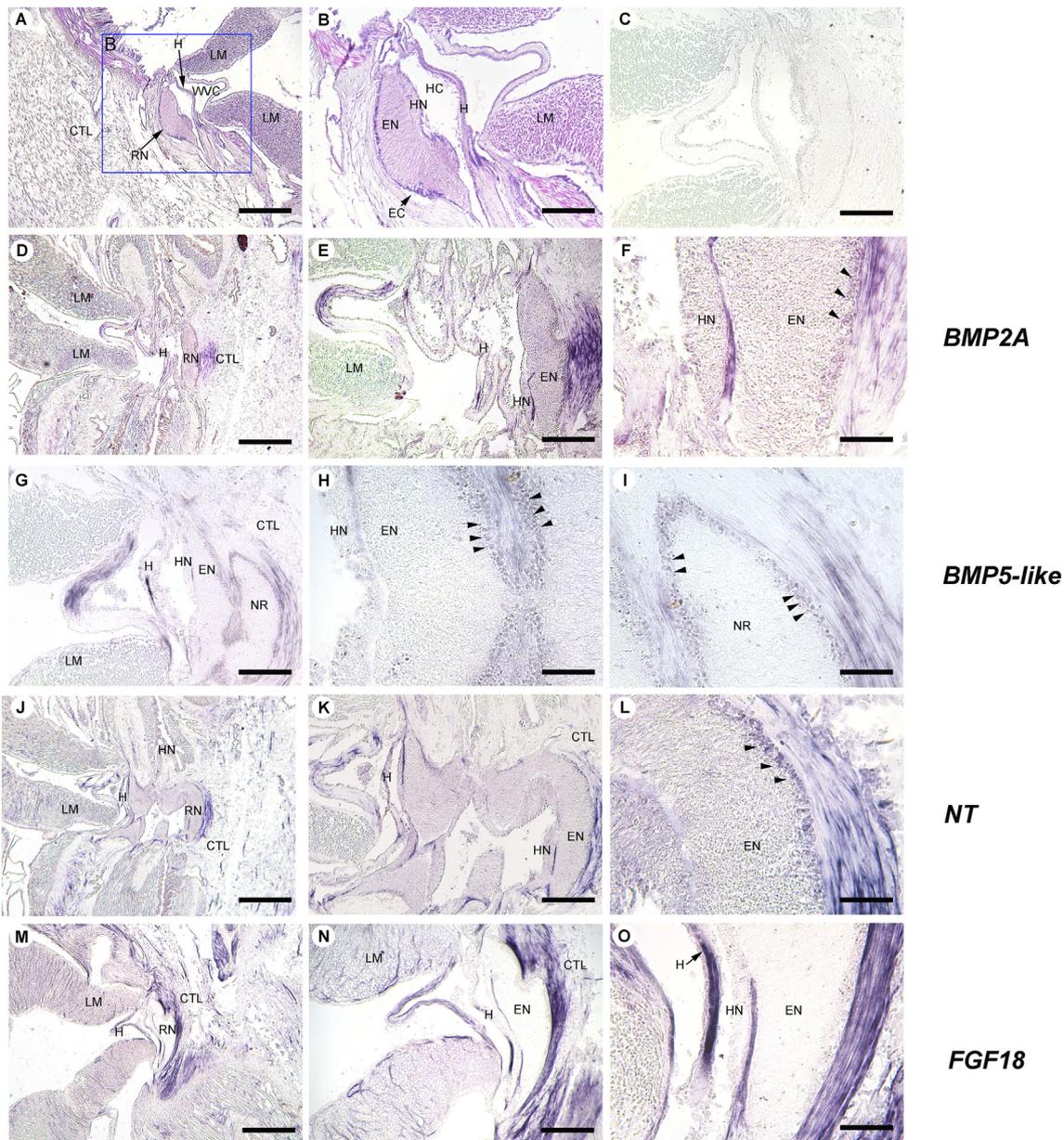
Figure 9. Histology and *in situ* hybridization of *H. scabra BMP 2A, BMP5-like, NT*, and *FGF18* in *H. scabra* radial nerve cord and other structures of the inner part body wall. **(A)** Cross-section of the radial nerve cord (RN) with the inner part of the body wall (IW) stained with H&E, showing the anatomy of the RN, the longitudinal muscle band (LM), the water vascular canal (WVC), the hemal lacuna (H) and the connective tissue layer (CTL) of the body wall. **(B)** High magnification of the box in (A). **(C)** Negative control micrograph using a DIG-labeled sense-strand riboprobe showing no positive signal. **(D-F)** An anti-sense *H. scabra BMP2A* riboprobe showing positive signals in the area of RN, ectoneural neuroepithelium (EN), CTL and H, the positive staining the cells of EN indicated by the arrows. **(G-I)** An anti-sense *H. scabra BMP5-like* riboprobe showing the positive signals in the RN, NR and the connective tissue connecting the LM, the positive staining the cells of EN and NR indicated by the arrows. **(J-L)** An anti-sense *H. scabra NT* riboprobe showing positive signals in the RN and CTL; the positive staining in cells of the EN are indicated by arrows. **(M-O)** Low to high magnifications of anti-sense *H. scabra FGF18* showing positive signals in the RN, H and CTL. Scale bars: 500 μm in A, D, J, and M; 200 μm in B, C, E, G, K and N; 100 μm in F, H, I, L and O.

found in several tissues. From *in situ* hybridization, *H. scabra BMP2A* and *BMP5-like* were expressed specifically in cells of the RN cord and CT layer of the body wall. This data is consistent to a previous study showing high levels of expression of *myostatin* in the body wall of *A. japonicus* [18]. It was suggested that myostatin may be involved in the growth of *A. japonicus*. The glial cells in the RN cord of *H. glaberrima* have been reported to be activated upon the requirement for neural repair [62]. Which may, in turn, regulate regeneration of the inner part of the body wall after injury [20]. The studies in *H. glaberrima* and *A. japonicus* have additionally shown the upregulation of TGF-β superfamily genes during

regeneration of visceral organs, radial nerve, and coelomocytes after evisceration [21, 43, 60].

Neurotrophins, as well as their receptor, were identified and characterized from *H. scabra,* and this was the first report of NTs in sea cucumbers. Phylogenetic tree analysis demonstrated that *H. scabra* and *S. purpuratus* NTs were closely related. We also found that echinoderm NTs formed a distinct clade which was rooted with vertebrates NT3, NGF, and BDNF. This was similar to the evolutionary analysis of *S. purpuratus* NT in the previous study [63]. The neurotrophins displayed six conserved cysteine regions, supporting a role for these in proper folding which

allows preferential interaction of neurotrophin precursors with a p75^{NTR}/sortilin receptor complex [64]. Similar to *BMP2A* and *BMP5-like* expression, *H. scabra NT* was detected in the cells of the RN cord and CT layer of the body wall. However, localization was specific to cells in the apical region of the neuroepithelium, an area that has been shown to contain neural progenitor cells responsible for neurogenesis in *H. glaberrima* [65]. Therefore, we propose that the *H. scabra* NT is an excellent candidate for regulating the generation of new cell in their CNS.

A transcript belonging to the fibroblast growth factor (FGF) family was identified in *H. scabra*. *H. scabra FGF18* was detected in *H. scabra* body wall and part of the RN cord. A *H. scabra* FGF receptor was also identified, supporting the FGF18 signaling in *H. scabra*. The occurrence of FGFs in various animal taxa indicates that this growth factor family appeared early in the bilaterian lineage and with profound conservation. *H. scabra FGF18* was extensively expressed in the CT layer of the body wall. In the brittle stars *Amphiura filiformis* [66] and the sea urchin *S. purpuratus*, the FGF has been proposed to be involved in skeletogenesis [67]. In humans, the FGF family contributes to the regulation of organogenesis and is widely associated with tissue repair and regeneration [68]. Hence, it was likely that *H. scabra* FGF18 might associate with cellular activity of the body wall.

5. Conclusions

This study provided a foundation analysis of genes encoding growth factors and their related receptors in *H. scabra*. Further analyses of *H. scabra* growth factor candidates (TGF-β2, BMP2, BMP2/4, BMP5-like, myostatin, inhibin, activin, NT, and FGF18), including sequence validation and comparative phylogenetics, suggested their evolutionary conservation among echinoderms as well as bilaterians. Spatial gene expression of selected growth factors by RT-PCR and *in situ* hybridization suggested that growth factors might be involved in the cellular activity of the RN cord and body wall. Collectively, this provided fundamental data for further study into regeneration in sea cucumbers and, potentially, regenerative medicine therapies.

Declarations

Author contribution statement

Napamanee Kornthong, Saowaros Suwansa-ard: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Teva Phanaksri: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data.

Jirawat Saetan, Supawadee Duangprom, Buranee Lekskul, Tipok Vivattanasarn, Sineenart Songkoomkrong, Prapaporn Jattujan: Performed the experiments.

Scott F. Cummins, Prasert Sobhon: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Funding statement

Data availability statement

Data will be made available on request.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

Supplementary content related to this article has been published online at https://doi.org/10.1016/j.heliyon.2021.e08370.

Acknowledgements

References

[1] C. Conand, Expansion of global sea cucumber fisheries buoys exports, Rev. Biol. Trop. 65 (2017) 1–10.

[2] J.F. Hamel, C. Conand, D.L. Pawson, A. Mercier, Advances in Marine Biology 129-223, Academic Press, 2001.

[3] T. Wang, L. Sun, M. Chen, in: H. Yang, J.F. Hamel, A. Mercier (Eds.), Developments in Aquaculture and Fisheries Science, Elsevier, 2015, pp. 177–209.

[4] R. Pangestuti, Z. Arifin, Medicinal and health benefit effects of functional sea cucumbers, J. Tradit. Complement. Med. 8 (2018) 341–351.

[5] A.L. Rychel, B.J. Swalla, Stem Cells in Marine Organisms (eds Baruch Rinkevich & Valeria Matranga) 245-265, Springer Netherlands, 2009.

[6] J.E. San Miguel-Ruiz, J.E. García-Arrarás, Common cellular events occur during wound healing and organ regeneration in the sea cucumber *Holothuria glaberrima*, BMC Dev. Biol. 7 (2007) 115.

[7] S.A. Bello, et al., Insights into intestinal regeneration signaling mechanisms, Dev. Biol. 458 (2020) 12–31.

[8] I.Y. Dolmatov, T.T. Ginanova, Muscle regeneration in holothurians, Microsc. Res. Tech. 55 (2001) 452–463.

[9] V.S. Mashanov, J.E. Garcia-Arrarás, Gut regeneration in holothurians: a snapshot of recent developments, Biol. Bull. 221 (2011) 93–109.

[10] J.E. García-Arrarás, et al., Regeneration of the enteric nervous system in the sea cucumber *Holothuria glaberrima*, J. Comp. Neurol. 406 (1999) 461–475.

[11] J.E. García-Arrarás, M.J. Greenberg, Visceral regeneration in holothurians, Microsc. Res. Tech. 55 (2001) 438–451.

[12] J.E. García-Arrarás, et al., Cellular mechanisms of intestine regeneration in the sea cucumber, *Holothuria glaberrima* Selenka (Holothuroidea:Echinodermata), J. Exp. Zool. 281 (1998) 288–304.

[13] V.S. Mashanov, I.Y. Dolmatov, Regeneration of digestive tract in the pentactulae of the far-eastern holothurian Eupentacta fraudatrix (Holothuroidea, Dendrochirota), Invertebr. Reprod. Dev. 39 (2001) 143–151.

[14] V.S. Mashanov, O.R. Zueva, J.E. Garcia-Arraras, Expression of Wnt9, TCTP, and Bmp1/Tll in sea cucumber visceral regeneration, Gene Expr. Patterns 12 (2012) 24–35.

[15] C. Rojas-Cartagena, et al., Distinct profiles of expressed sequence tags during intestinal regeneration in the sea cucumber *Holothuria glaberrima*, Physiol. Genom. 31 (2007) 203–215.

[16] P. Santiago, J.L. Roig-López, C. Santiago, J.E. García-Arrarás, Serum amyloid A protein in an echinoderm: its primary structure and expression during intestinal regeneration in the sea cucumber *Holothuria glaberrima*, J. Exp. Zool. 288 (2000) 335–344.

[17] E.C. Suárez-Castillo, W.E. Medina-Ortiz, J.L. Roig-López, Garcia-Arraras, J.E. Ependymin, A gene involved in regeneration and neuroplasticity in vertebrates, is overexpressed during regeneration in the echinoderm *Holothuria glaberrima*, Gene 334 (2004) 133–143.

[18] S. Li, et al., Molecular characterization, expression analysis of the myostatin gene and its association with growth traits in sea cucumber (*Apostichopus japonicus*), Comp. Biochem. Physiol. B Biochem. Mol. Biol. 201 (2016) 12–20.

[19] Z. Lv, et al., VEGF-like protein from *Apostichopus japonicus* promotes cell proliferation and migration, Dev. Comp. Immunol. 92 (2019) 230–237.

[20] L. Sun, et al., Large scale gene expression profiling during intestine and body wall regeneration in the sea cucumber *Apostichopus japonicus*, Comp. Biochem. Physiol. Genom. Proteomics 6 (2011) 195–205.

[21] L. Sun, H. Yang, M. Chen, D. Ma, C. Lin, RNA-Seq reveals dynamic changes of gene expression in key stages of intestine regeneration in the sea cucumber *Apostichopus japonicus*, PLoS One 8 (2013) e69441.

[22] P.A. Ortiz-Pineda, et al., Gene expression profiling of intestinal regeneration in the sea cucumber, BMC Genom. 10 (2009) 262.

[23] M. Atanasova, A. Whitty, Understanding cytokine and growth factor receptor activation mechanisms, Crit. Rev. Biochem. Mol. Biol. 47 (2012) 502–530.

[24] R. Goldman, Growth factors and chronic wound healing: past, present, and future, Adv. Wound Care 17 (2004) 24–35.

[25] A.T. Grazul-Bilska, et al., Wound healing: the role of growth factors, Drugs Today 39 (2003) 787–800.

[26] A. Bafico, S. Aaronson, Classification of growth factors and their receptors, in: D.W. Kufe, R.E. Pollock, R.R. Weichselbaum, et al. (Eds.), Cancer Medicine, BC Decker, 2003.

[27] M. Fainzilber, et al., CRNF, a molluscan neurotrophic factor that interacts with the p75 neurotrophin receptor, Science 274 (1996) 1540–1543.

[28] K. Homma, T. Matsushita, S. Natori, Purification, characterization, and cDNA cloning of a novel growth factor from the conditioned medium of NIH-Sape-4, an embryonic cell line of *Sarcophaga peregrina* (flesh fly), J. Biol. Chem. 271 (1996) 13770–13775.

[29] G. Kutty, et al., Identification of a new member of transforming growth factor-beta superfamily in Drosophila: the first invertebrate activin gene, Biochem. Biophys. Res. Commun. 246 (1998) 644–649.

[30] E. Legac, G.L. Vaugier, F. Bousquet, M. Bajelan, M. Leclerc, Primitive cytokines and cytokine receptors in invertebrates: the sea star *Asterias rubens* as a model of study, Scand. J. Immunol. 44 (1996) 375–380.

[31] Q.R. Liu, et al., A developmental gene (*Tolloid*/BMP-1) is regulated in Aplysia neurons by treatments that induce long-term sensitization, J. Neurosci. 17 (1997) 755–764.

[32] B.J. Rutledge, K. Zhang, E. Bier, Y.N. Jan, N. Perrimon, The Drosophila spitz gene encodes a putative EGF-like growth factor involved in dorsal-ventral axis formation and neurogenesis, Genes Dev. 6 (1992) 1503–1517.

[33] A.B. Smit, et al., Growth-controlling molluscan neurons produce the precursor of an insulin-related peptide, Nature 331 (1988) 535–538.

[34] P. Stenzel, L.M. Angerer, B.J. Smith, R.C. Angerer, W.W. Vale, The univin gene encodes a member of the transforming growth factor-beta superfamily with restricted expression in the sea urchin embryo, Dev. Biol. 166 (1994) 149–158.

[35] D. Sutherland, C. Samakovlis, M.A. Krasnow, Branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching, Cell 87 (1996) 1091–1101.

[36] K.A. Wharton, G.H. Thomsen, W.M. Gelbart, Drosophila 60A gene, another transforming growth factor beta family member, is closely related to human bone morphogenetic proteins, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 9214–9218.

[37] W.C. Wildering, P.M. Hermann, A.G. Bulloch, Lymnaea epidermal growth factor promotes axonal regeneration in CNS organ culture, J. Neurosci. 21 (2001) 9345–9354.

[38] S.H. Crossman, S.J. Streichan, J.P. Vincent, EGFR signaling coordinates patterning with cell survival during Drosophila epidermal development, PLoS Biol. 399865 (2018).

[39] L. Du, et al., Unique patterns of organization and migration of FGF-expressing cells during Drosophila morphogenesis, Dev. Biol. 427 (2017) 35–48.

[40] A.K. Lochab, C.G. Extavour, Bone Morphogenetic Protein (BMP) signaling in animal reproductive system development and function, Dev. Biol. 427 (2017) 258–269.

[41] F. Lapraz, et al., RTK and TGF-β signaling pathways genes in the sea urchin genome, Dev. Biol. 300 (2006) 132–152.

[42] Z. Sun, C.A. Ettensohn, TGF-β sensu stricto signaling regulates skeletal morphogenesis in the sea urchin embryo, Dev. Biol. 421 (2017) 149–160.

[43] V.S. Mashanov, O.R. Zueva, J.E. García-Arrarás, Transcriptomic changes during regeneration of the central nervous system in an echinoderm, BMC Genom. 15 (2014).

[44] Y. Zhao, H. Yang, K.B. Storey, M. Chen, RNA-seq dependent transcriptional analysis unveils gene expression profile in the intestine of sea cucumber *Apostichopus japonicus* during aestivation, Comp. Biochem. Physiol. Genom. Proteonomics 10 (2014) 30–43.

[45] Q. Gao, et al., Transcriptome analysis and discovery of genes involved in immune pathways from coelomocytes of sea cucumber (*Apostichopus japonicus*) after *Vibrio splendidus* challenge, Int. J. Mol. Sci. 16 (2015) 16347–16377.

[46] X. Wu, et al., Transcriptomic analysis of sea cucumber (*Holothuria leucospilota*) coelomocytes revealed the echinoderm cytokine response during immune challenge, BMC Genom. 21 (2020) 306.

[47] J.G. Medina-Feliciano, et al., Draft genome of the sea cucumber *Holothuria glaberrima*, a model for the study of regeneration, Front. Mar. Sci. 8 (2021) 603410.

[48] N. Kornthong, et al., The effect of sea cucumber extract (*Holothuria scabra*) on the proliferation of human placenta derived mesenchymal stromal cells, J. Med. Assoc. Thai. 103 (2020) 24–31.

[49] I.Y. Dolmatov, T.T. Ginanova, Post-autotomy regeneration of respiratory trees in the holothurian *Apostichopus japonicus* (Holothuroidea, Aspidochirotida), Cell Tissue Res. 336 (2009) 41–58.

[50] C. Franco, et al., Understanding regeneration through proteomics, Proteomics 13 (2013) 686–709.

[51] S.A. Mitu, et al., Evidence for a saponin biosynthesis pathway in the body wall of the commercially significant sea cucumber *Holothuria scabra*, Mar. Drugs 15 (2017) 349.

[52] S. Suwansa-ard, et al., Transcriptomic discovery and comparative analysis of neuropeptide precursors in sea cucumbers (Holothuroidea), Peptides 99 (2018) 231–240.

[53] G.E. Crooks, G. Hon, J.M. Chandonia, S.E. Brenner, WebLogo: a sequence logo generator, Genome Res. 14 (2004) 1188–1190.

[54] G. Stecher, K. Tamura, S. Kumar, Molecular evolutionary genetics analysis (MEGA) for macOS, Mol. Biol. Evol. 37 (2020) 1237–1239.

[55] N. Kornthong, et al., Molecular characterization of a vitellogenesis-inhibiting hormone (VIH) in the mud crab (*Scylla olivacea*) and temporal changes in abundances of VIH mRNA transcripts during ovarian maturation and following neurotransmitter administration, Anim. Reprod. Sci. 208 (2019).

[56] S. Duangprom, et al., Identification and expression of prostaglandin E synthase (PGES) gene in the central nervous system and ovary during ovarian maturation of the female mud crab, *Scylla olivacea*, Anim. Reprod. Sci. 198 (2018) 220–232.

[57] N. Kornthong, et al., Neuronal classification and distribution in the central nervous system of the female mud crab, *Scylla olivacea*, Microsc. Res. Tech. 77 (2014) 189–200.

[58] G. Tettamanti, et al., Growth factors and chemokines: a comparative functional approach between invertebrates and vertebrates, Curr. Med. Chem. 13 (2006) 2737–2750.

[59] W.E. Mayer, et al., Isolation and characterization of lymphocyte-like cells from a lamprey, Proc. Natl. Acad. Sci. U. S. A. 99 (2002) 14350–14355.

[60] W. Shi, et al., Transcriptome analysis of sea cucumber (*Apostichopus japonicus*) polian vesicles in response to evisceration, Fish Shellfish Immunol. 97 (2020) 108–113.

[61] F. Zito, et al., Expression of univin, a TGF-β growth factor, requires ectoderm–ECM interaction and promotes skeletal growth in the sea urchin embryo, Dev. Biol. 264 (2003) 217–227.

[62] V.S. Mashanov, O.R. Zueva, J.E. Garcia-Arraras, Radial glial cells play a key role in echinoderm neural regeneration, BMC Biol. 11 (2013) 49.

[63] R.D. Burke, et al., A genomic view of the sea urchin nervous system, Dev. Biol. 300 (2006) 434–460.

[64] H.K. Teng, et al., ProBDNF induces neuronal apoptosis via activation of a receptor complex of p75NTR and sortilin, J. Neurosci. 25 (2005) 5455–5463.

[65] V.S. Mashanov, O.R. Zueva, J.E. García-Arrarás, Heterogeneous generation of new cells in the adult echinoderm nervous system, Front. Neuroanat. 9 (2015) 123.

[66] A. Czarkwiani, D.V. Dylus, L. Carballo, P. Oliveri, FGF Signalling Plays Similar Roles in Development and Regeneration of the Skeleton in the Brittle star *Amphiura Filiformis*, Development, 2019, p. 632968.

[67] E. Rottinger, et al., FGF signals guide migration of mesenchymal cells, control skeletal morphogenesis and regulate gastrulation during sea urchin development, Development 135 (2008) 353–365.

[68] Q.M. Nunes, Y. Li, C. Sun, T.K. Kinnunen, D.G. Fernig, Fibroblast growth factors as tissue repair and regeneration therapeutics, PeerJ 4 (2016) e1535.