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

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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/organisms, 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
characterized [7, 15, 16, 17], which include genes in the growth factor family, such as myostatin and vascular endothelial growth factor (VEGF) [20, 21, 22], where they may regulate organ regeneration. Moreover, bone morphogenetic proteins (BMPs) are superfamily ligands, which have been shown to increase during regeneration in Apostichopus japonicus and H. glaberrima [20, 21, 22], where they may regulate organ regeneration. Growth factors can be grouped into families of molecules having 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.

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'-TACGGAAGACAGCACACAAAGACG-3' | 299 |
| | BMP2AR1 | Reverse | 5'-TGGCCAGGAAATGGGACACTTGCG-3' | |
| BMP2/4 | BMP2/4F | Forward | 5'-CGGAGGAGGAGGTCAAGGGTC-3' | 166 |
| | BMP2/4R | Reverse | 5'-GCTGTTAAGACATGGCGGGCGG-3' | |
| BMP5-like | BMP5likeF | Forward | 5'-CAAGTACAGAAGTGCCTGACAGG-3' | 298 |
| | BMP5likeR1 | Reverse | 5'-CCCACTTTATGGGGCCGAAAC-3' | |
| Inhibin | InhibinF | Forward | 5'-CCATGTGACTGCGCGGTGAGG-3' | 251 |
| | InhibinR1 | Reverse | 5'-CCAGTTGGAATAGGCTGACCAGT-3' | |
| Myostatin | MISTNF | Forward | 5'-GATGCCAGTCCACAGGACCTC-3' | 286 |
| | MISTNR1 | Reverse | 5'-GGGGTATTGAGGACGATACTATGCG-3' | |
| Activin | ActivinF | Forward | 5'-CTGACGATCTGATATAGTAG-3' | 326 |
| | ActivinR | Reverse | 5'-CGATTCTCTATGGGGGATT-3' | |
| TGFbeta2 | TGF2F | Forward | 5'-CACCAGGAACAACACACTCGGAG-3' | 350 |
| | TGF2R1 | Reverse | 5'-CGGAGAACGCAACGGGTAGGCG-3' | |
| FGF18 | FGF1F | Forward | 5'-CCGAGCAGTFCAGGCACAAATACC-3' | 200 |
| | FGF1R | Reverse | 5'-CTGCTTCAAGAGATGCTCCC-3' | |
| Neurotrophin | NTF | Forward | 5'-TGGGTAGAAGAATGGCATGAC-3' | 152 |
| | NTR | Reverse | 5'-GAACACGATTGCCAGGACAT-3' | |
| H. scabra 16S mitochondrial rRNA | 16SF | Forward | 5'-GAAAGGAGGACACGACCTGCG-3' | 187 |
| | 16SR | Reverse | 5'-CTTTCGATACGATCTGCGTC-3' | |

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

| Name of transcript | Primer | Direction | Nucleotide sequence |
|--------------------|--------|-----------|--------------------|
| BMP2/4 | BMP2/4F | 3RACE | 5'-GGAAATGACTGATGCGTCGTCGTCG-3' |
| | BMP2/4R | 5RACE | 5'-TCGGCGCTGGGCGGCGCTTCCTCTCA-3' |
| BMP2A | BMP2AF3 | 3RACE | 5'-GGAGGAGGAGAGGTCAAGGGTCGTCG-3' |
| | BMP2AR5 | 5RACE | 5'-CCAGGCGGAGGAGGTCAAGGGTCGTCG-3' |
| BMP5-like | BMP5F3 | 3RACE | 5'-TCGATGCTGGCAGCGGCAGGCGG-3' |
| | BMP5R5 | 5RACE | 5'-CCAGCAATTCTTCGCTTCCTATCCC-3' |
| Inhibin | InhibinF3 | 3RACE | 5'-CTCAACATCATTGCGCTTCTGCG-3' |
| | InhibinR5 | 5RACE | 5'-GACGAAGACCGGAGGTCAAGGGTCGTCG-3' |
| Myostatin | MISTNF3 | 3RACE | 5'-CCAGATGGCAGGAGGTCAAGGGTCGTCG-3' |
| | MISTNR5 | 5RACE | 5'-GGTGGATTATCAACAACACTGGC-3' |
| Activin | ActivinF3 | 3RACE | 5'-CGGAGGAGGAGAGGTCAAGGGTCGTCG-3' |
| | ActivinR5 | 5RACE | 5'-TACGGAAGACAGCACACAAAGACG-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-NF | 0 | 0 |
| BDNF | 0 | 0 |
| FGF18 | 1 | 1 |
| IGFs | 1 | 0 |
| VEGFs | 1 | 1 |
| EGF | 1 | 1 |
| Ependymin-related | 1 | 0 |
| Uniniv | 0 | 0 |
| Cryptic | 0 | 0 |
| BMP receptor-type II | 4 | 3 |
| Activin receptor | 1 | 1 |
| Tyrosine kinase receptor | 1 | 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.

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
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.uifl.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. Bilateral 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.
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 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; Musma, Mus musculus; Sacko, Saccoglossus kowalevskii; Logi, Lottia gigantea; Apoja, Apostichopus japonicus; Lytva, Lytechinus variegatus; Strpu, Strongylocentrotus purpuratus; Acapl, Acanthaster planci; Holigl, Holothuria glaberrima; Holsc, Holothuria scabra. Accession numbers of TGF-β2 protein sequences presented are provided in Supplement data 4.
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 to produce DIG-labeled sense-strand (M13 forward (5'-AACAGCTATGACCATG-3')) and anti-sense RNA riboprobes (DIG-RNA labeling kit SP6/T7, Roche, Germany). Recombinant plasmids containing *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 reaction (RT-PCR) to proceed. Controls. When appropriate, reactions without cDNA were 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 then rehydrated in H₂O, and 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'-AAGCTATGACCATG-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...
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 tRNA, 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 FGF/17/18 families (Figure 1 and Supplement Data 2 and 3). The members of the growth factors in the TGF-β, NT and FGF/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. Mean-
BMP2 and BMP4, cephalochordates and hemichordates BMP2/4, and H. scabra chordates, hemichordates, molluscs, insects, crustaceans, and other compared to other BMP2, BMP4 and BMP2/4 in other species from BMP2/4 of other echinoderm species, 42 representative species from chordates, hemichordates, molluscs, insects, A. japonicus S. purpuratus TGF-β H. scabra representatives) FGF8/17/18 cluster (Figure 7B). 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 NTs, 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.

3.1.2. Neurotrophin (NT) A sequence of 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.

3.1.3. 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 plancii FGF18, and 29% similarity to the lancelets, Branchiostoma floridae and Branchiostoma lanceolatum FGF8/17/18 proteins. H. scabra FGF18 shared 27% similarity to molluscs, Miothopeuctus 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 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 plancii 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 BMP2A/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 BMP2 from molluscan, insect and crustacean representative species (Figure 3A, Supplement Data 5). H. scabra BMP2A protein showed 83% similarity with H. glaberrima BMP2A, 28% 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 inhibitin showed 71% similarity with H. glaberrima inhibitin and 42% similarity to A. japonicus inhibitin C, it also displayed 20–24% similarity with chordates, insects, and crustaceans inhibitin (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 inhibitin 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.

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, inhibitin, activin, BMP2, BMP2/4 and BMP5-like that were found in this study.
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 Cs 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 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; Aplica, 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 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; Brfl, Branchiostoma floridae; Lytva, Lytechinus variegatus; Ampho, Amphipholis kochii; Mitye, Mitxopesten yessoensis; Strpu, Strongylocentrotus purpuratus; Astsa, Asterias rubens; Acapl, Acanthaster planci; Holgl, Holothuria glaberrima; Holsc, Holothuria scabra. Accession numbers of FGF protein sequences presented are provided in Supplement data 8.
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-β 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 hybridisation

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 BMP2/4 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 BMP2A 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 RN 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 RN 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 RN 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
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

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**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.
allows preferential interaction of neurotrophin precursors with a p75NTR/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 [67]. 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-arud: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

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

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

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

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**Data availability statement**

Data will be made available on request.

**Declaration of interests statement**

The authors declare no conflict of interest.

**Additional information**

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Data will be made available on request.
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