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Contribution of the TGFβ signaling pathway to pigmentation in sea cucumber (*Apostichopus japonicus*)

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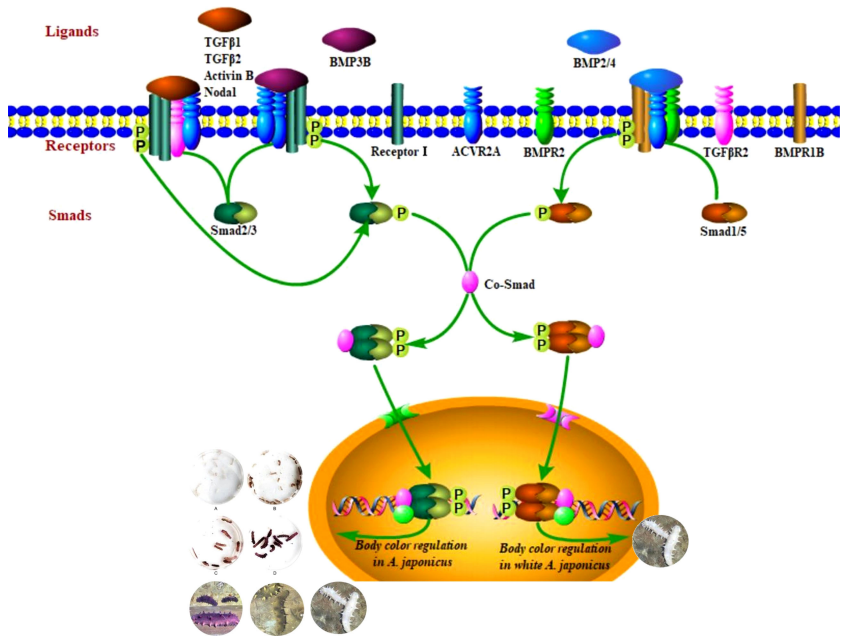
Pigmentation mediated by the transforming growth factor β (TGFβ) signaling pathway is a key trait for understanding environmental adaptability and species stability. In this study, TGFβ signaling pathway members and their expression patterns in different color morphs of the sea cucumber *Apostichopus japonicus* were evaluated. Using a bioinformatics approach, 22 protein sequences of TGFβ signaling pathway members in *A. japonicus* were classified, including 14 that were identified for the first time in the species, including 7 ligands, 6 receptors, and 1 R-Smad. We further evaluated mRNA expression data for different color morphs and pigmentation periods. These results support the hypothesis that both subfamilies of the TGFβ superfamily, i.e., the TGFβ/activin/Nodal and BMP/GDF/AMH subfamilies, are involved in the regulation of pigmentation in *A. japonicus*. The former subfamily was complete and contributes to the different color morphs. The BMP/GDF/AMH subfamily was incomplete. BMP2/4-induced differentiation of white adipocytes was regulated by the BMP2/4–ACVR2A–Smad1 signaling pathway. These findings provide insight into the TGFβ family in early chordate evolution as well as the molecular basis of color variation in an economically valuable species.

KEYWORDS

TGFβ signaling pathway, regulatory mechanism, pigmentation, *Apostichopus japonicas*, gene family divergence



GRAPHICAL ABSTRACT

1 Introduction

The sea cucumber *Apostichopus japonicus* is a commercially important marine species in China (Chen et al., 2022). Color variation, one of the most important characteristics of *A. japonicus*, plays a significant role in determining market price (Kang et al., 2011) and is an important trait for breeding. In China, this species is mainly green, and purple and white morphs are very rare and highly valuable (Bai et al., 2016). Extensive studies have shown that the growth and development of sea cucumber are affected by various environmental factors, such as temperature and salinity (Chen et al., 2007; Wang et al., 2007; Ji et al., 2008). There are significant differences in the tolerance of sea cucumbers with different body colors to environmental factors (Bao, 2008; Guo et al., 2020; Li et al., 2020). For example, purple *A. japonicus* has a wider temperature range and stronger salt tolerance, while the white morph has a higher temperature tolerance but narrower range of salinity tolerance than those of the green morph (Zhao et al., 2018; Zhu et al., 2013).

Pigmentation is a tractable and relevant trait for understanding key issues in evolutionary biology such as adaptation, speciation and the maintenance of balanced polymorphisms (Henning et al., 2013).Substantial recent research has focused on the identification of genetic pathways that determine pigmentation variation (Hubbard et al., 2010; Henning et al., 2013). Studies of animal models have found that the TGFβ signaling pathway mediates many biological processes, such as pigmentation, tissue and organ development, and stress resistance (Cheng, 2008; Hubbard et al., 2010). Recent structural, biochemical, and cellular studies have provided significant insight into the mechanisms underlying TGFβ signaling. In brief, a TGFβ ligand initiates signaling by binding to and bringing together type I and type II receptor on the cell surface. This allows receptor II to phosphorylate receptor I, which then regulates target gene expression by the phosphorylation of Smad proteins. The number and type of TGFβ family members have been evaluated in model organisms, ranging from worms and flies to mammals (Massagué and Chen, 2000; Patterson and Padgett, 2000; ten Dijke et al., 2000). Six conserved cysteine residues characteristic of the TGFβ family are encoded by 6 open reading frames in worms, 9 in flies, and 42 in humans (Linton et al., 2001).

Although studies of the TGFβ family in non-model organisms are increasing, relatively little is known about functional changes and divergence in expression patterns between invertebrates and vertebrates (Lapraz et al., 2007; Weiss and Attisano, 2013; Zheng et al., 2018). Echinoderms, which first appeared in the early Cambrian period (Bottjer et al., 2006), occupy a critical phylogenetic position for understanding the origin of chordates (Lowe et al., 2015). The radiation of echinoderms was believed to be responsible for the Mesozoic Marine Revolution (Signor and Brett, 1984). In particular, sea cucumbers are an outstanding representative of the phylum, as they have survived ice ages and are considered “living fossils” (Bottjer et al., 2006). Despite the importance of pigmentation mediated by the TGFβ signaling pathway (Cheng, 2008; Hubbard et al., 2010; Henning et al., 2013), few studies have evaluated the TGFβ signaling pathway in sea cucumbers. Only 14 ligands (some sharing the same name), 6 receptors, and 2 R-Smads have been recorded in GenBank. In

addition, some loci have informal names, such as Sj-BMP2/4 (accession no. PIK56114.1 and BAC53989.1), and some were not classified in detail, e.g., putative TGFβ (accession no. PIK61515.1). Accordingly, their functions and roles in morphs with different body colors are unclear. This can be explained, in part, by poor sampling of genomes (Sodergren et al., 2006; Cameron et al., 2015; Hall et al., 2017; Sun et al., 2017; Zhang et al., 2017). In this study, the types and quantities of TGFβ signaling pathway members in *A. japonicus* were characterized for the first time and expression levels in different color morphs and developmental stages were evaluated, providing an important basis for analyses of functions of TGFβ signaling in invertebrates.

2 Materials and methods

2.1 Sequence analysis

All TGFβ ligand receptors and Smad protein sequences of *A. japonicus* available on NCBI were obtained and compared using BLAST (Basic Local Alignment Search Tool) (Tables 1–3). Multiple sequence alignments were analyzed using the ClustalW Multiple Alignment program (http://www.ebi.ac.uk/clustalw/). Separate trees were generated based on ligand, receptor, and SMAD amino acid sequences using the neighbor-joining (NJ) algorithm within MEGA version 7.0. The reliability of the tree was assessed by 1000 bootstrap repetitions.

2.2 Animals

2.2.1 Sea cucumbers of different color morphs

Healthy sea cucumbers aged 2 years and weighing 120 ± 10 g were collected from green, purple, and white cultivated populations (Figure 1). The purple and white morphs are genetically stable and have been bred by our research team for nearly 20 years.

2.2.2 Purple sea cucumber at different developmental stages

9 purple sea cucumbers with a body weight of >180 g were screened as the parent population. Artificial labor was stimulated by drying in the shade and running water (20.5°C). Male individuals were removed from the incubator immediately after ejaculation. All parents were removed when the egg density was 20–30 eggs/mL. Then, the water temperature was increased to 21.0 ± 0.2°C for incubation. During the incubation period, the incubator was agitated once an hour, and micro-aerated continuously for 24 h to ensure an even distribution of fertilized eggs. Marine red yeast was fed to early auricularia after hatching. When 10% to 20% of doliolaria formed, a corrugated plate frame after disinfection was placed as the attachment matrix. After the larvae were attached, they were gradually transitioned to artificial compound feed. The feeding amount was 0.5% to 2% of the body weight. Juveniles were randomly selected every 3 days after the larvae developed to pentactula and were placed in a Petri dish to observe the change in body color. The pigmentation stage was defined at the point at which 80% of individuals were completely pigmented.

2.3 mRNA expression of TGFβ signaling pathway genes mRNA in *A. japonicas*

9 body walls from each sample of different color morphs and purple sea cucumber at different developmental stages were peeled away carefully, flash-frozen in liquid nitrogen, and stored at −80°C for subsequent total RNA extraction. Specific primers for BMP2/4, ACVR2A, Smad1, TGFβR2, Smad2/3, and grb2 (a housekeeping gene used as an internal reference) based on known *A. japonicus* sequences (Table 4) were designed using Oligo 7.0. Primers were synthesized by Invitrogen Biotechnology Co., Ltd. (Shanghai, China). TRIzol Reagent was used to isolate total RNA from the body walls according to the manufacturer's instructions (Invitrogen, Waltham, MA, USA) and contaminating genomic DNA was eliminated using RNase-free DNase (Takara, Tokyo, Japan). The RNA samples were reverse-transcribed using the Prime Script RT-PCR Kit (Takara, Tokyo, Japan). Equal amounts of cDNA were used for real-time quantitative RT-PCR using in a PikoReal 96-well RT-PCR System (Thermo Scientific., Waltham, MA, USA). Amplification was performed in a total volume of 10 μL, containing 5 μL of 2× SYBR Green master Mix, 1 μL of diluted cDNA, 0.4 μL of each primer, and 3.2 μL of PCR-grade water. The PCR cycling conditions were 95°C for 5 min followed by 35 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 1 min, and a final elongation step at 72°C for 7 min. Each sample was run in triplicate along with the internal control gene (grb2). The PCR products were visualized on a UV-transilluminator after electrophoresis on a 1.5% agarose gel containing ethidium bromide.

2.4 Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0, and all data were assessed using one-way ANOVA. Differences in means between groups were assessed using Tukey's honestly significant difference test for *post hoc* multiple comparisons. All data are expressed as the mean ± standard deviation (SD). Values of $p < 0.05$ indicated a statistically significant difference.

3 Results

3.1 Phylogenetic analysis based on ligand sequences

In a phylogenetic tree based on amino acid sequences from multiple TGFβ ligands, the ligands of the same type formed clusters. The phylogenetic tree is shown in Figure 2 and the corresponding sequences are shown in Table 1. According to the phylogenetic tree, 14 known TGFβ ligands from *A. japonicus* were assigned to 7 classes: TGFβ1, TGFβ2, Nodal, Activin/Inhibin, BMP2/4, BMP3, and GDF8 (Growth Differentiation Factor 8, alternative name myostatin (de Caestecker, 2004)). Notably, TGFβ2 of *A. japonicus* was classified as TGFβ2 but was also closely related to TGFβ3. The BMP2/4 cluster contained BMP2A, BMP2, BMP, and Sj-BMP2/4 of *A. japonicus*. BMP3/3B of *A. japonicus* was classified into BMP3. Putative activin BX1 and putative inhibin beta C chain-like of *A. japonicus* were included in the Activin/Inhibin cluster.

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TABLE 1 Ligand protein sequences included in the present study.

Accession no.	Protein name	Species
PIK34829.1	**putative TGFβ1 like**	**Apostichopus japonicus**
QHG11580.1	**putative TGFβ1X1**	**Apostichopus japonicus**
PIK56215.1	**putative TGFβ1X1**	**Apostichopus japonicus**
XP_029964045.1	TGFβ1X2	Salarias fasciatus
XP_041850924.1	TGFβ1X1	Melanotaenia boesemani
XP_031614329.1	TGFβ1	Oreochromis aureus
XP_033486555.1	TGFβ1X1	Epinephelus lanceolatus
XP_040902844.1	TGFβ1	Toxotes jaculatrix
XP_033851930.2	TGFβ1X1	Acipenser ruthenus
XP_035241816.1	TGFβ1X1	Anguilla anguilla
XP_026865903.2	TGFβ1X2	Electrophorus electricus
XP_036440909.1	TGFβ1X1	Colossoma macropomum
KAG9273341.1	TGFβ1X1	Astyanax mexicanus
PIK45926.1	**BMP2A**	**Apostichopus japonicus**
PIK48439.1	**BMP2**	**Apostichopus japonicus**
PIK57098.1	**BMP**	**Apostichopus japonicus**
AAF19841.1	BMP2/4	Branchiostoma belcheri
QYF06707.1	BMP2/4	Holothuria scabra
PIK56114.1	**Sj-BMP2/4**	**Apostichopus japonicus**
BAC53989.1	**Sj-BMP2/4**	**Apostichopus japonicus**
AAD28038.1	BMP2/4	Lytechinus variegatus
ACA04460.1	BMP2/4	Strongylocentrotus purpuratus
ABG00199.1	BMP2/4	Paracentrotus lividus
BBC77411.1	BMP2/4	Temnopleurus reevesii
PIK37799.1	**BMP3/3B**	**Apostichopus japonicus**
KAF3695343.1	BMP3	Channa argus
XP_033946079.1	BMP3	Pseudochaenichthys georgianus
XP_007425424.1	BMP3	Python bivittatus
XP_042727338.1	BMP3	Lagopus leucura
XP_021252303.1	BMP3	Numida meleagris
PIK42868.1	**TGFβ family member nodal**	**Apostichopus japonicus**
ACF32774.1	Nodal	Heliocidaris erythrogramma
ACF32773.1	Nodal	Heliocidaris tuberculata
XP_036937551.1	Nodal2	Acanthopagrus latus
XP_034426535.1	Nodal2	Hippoglossus hippoglossus
KFM00388.1	Nodal	Aptenodytes forsteri
XP_035248296.1	Nodal	Anguilla anguilla
RXN30610.1	Nodal	Labeo rohita
QYF06711.1	GDF8	Holothuria scabra
AJQ81037.1	**GDF8**	**Apostichopus japonicus**

(Continued)

TABLE 1 Continued

Accession no.	Protein name	Species
XP_013394669.1	GDF8	Lingula anatina
XP_014253049.1	GDF8	Cimex lectularius
XP_046672106.1	GDF8	Homalodisca vitripennis
RWS12911.1	GDF8	Dinothrombium tinctorium
XP_023223240.1	GDF8	Centruroides sculpturatus
QYF06710.1	inhibin	Holothuria scabra
PIK34215.1	**putative inhibin beta C chain-like**	**Apostichopus japonicus**
QYF06712.1	activin	Holothuria scabra
PIK48233.1	**putative activin B X1**	**Apostichopus japonicus**
XP_037927328.1	INHβB	Teleopsis dalmanni
XP_022218905.1	INHβA	Drosophila obscura
XP_017154392.1	INHβA	Drosophila miranda
XP_002028363.1	INHβA	Drosophila persimilis
XP_033236864.1	INHβA	Drosophila pseudoobscura
QYF06713.1	TGFβ2	Holothuria scabra
PIK61515.1	**putative TGFβ2**	**Apostichopus japonicus**
XP_022090565.1	TGFβ2	Acanthaster planci
XP_038073348.1	TGFβ2	Patiria miniata
BCB62973.1	TGFβ	Patiria pectinifera
XP_041467929.1	TGFβ2	Lytechinus variegatus
XP_030855505.1	TGFβ2	Strongylocentrotus purpuratus
QAV52899.1	TGFβ	Mesocentrotus nudus
XP_041951915.1	TGFβ3	Alosa sapidissima
XP_042562890.1	TGFβ3	Clupea harengus
XP_039597176.1	TGFβ3	Polypterus senegalus
XP_028678165.1	TGFβ3	Erpetoichthys calabaricus
XP_042593951.1	TGFβ2	Cyprinus carpio
KAA0709699.1	TGFβ2	Triplophysa tibetana
XP_039388520.1	TGFβ2X2	Mauremys reevesii
XP_037751639.1	TGFβ2	Chelonia mydas
XP_005307005.1	TGFβ2	Chrysemys picta bellii
XP_003800184.1	TGFβ2X2	Otolemur garnettii
XP_008825028.1	TGFβ2	Nannospalax galili
CAA40672.1	TGFβ2	Mus musculus
XP_021016320.1	TGFβ2X2	Mus caroli

3.2 Phylogenetic analysis of receptors

In a phylogenetic tree based on amino acid sequences from multiple TGFβ receptors proteins, each type of receptor assembled in a cluster. The phylogenetic tree is shown in Figure 3 and corresponding sequences are shown in Table 2. According to the phylogenetic tree, six TGFβ receptors from *A. japonicus* were classified into six classes: TGFβR2 [transforming growth factor beta receptor 2, alternative name TβR2 (Hart et al., 2002)], TGFβR3 (transforming growth factor beta receptor 3, alternative name TβR3), BMPR1B [bone morphogenetic protein

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TABLE 2 Receptor protein sequences included in the present study.

Accession no.	Protein name	Species
XP_026886611.2	TβR2	Electrophorus electricus
TSK92904.1	TβR2	Bagarius yarrelli
XP_017324951.1	TβR2	Ictalurus punctatus
XP_026802727.2	TβR2	Pangasianodon hypophthalmus
PIK56848.1	**putative TβR2**	**Apostichopus japonicus**
XP_033116886.1	TβR2	Anneissia japonica
XP_030828751.1	TβR2	Strongylocentrotus purpuratus
XP_038058105.1	TβR2	Patiria miniata
XP_033624779.1	TβR2	Asterias rubens
PIK56867.1	**putative TβR3**	**Apostichopus japonicus**
CAC5417350.1	TβR3	Mytilus coruscus
CAG2237547.1	TβR3	Mytilus edulis
XP_038129033.1	TβR3	Cyprinodon tularosa
XP_040482976.1	TβR3X2	Ursus maritimus
XP_036901372.1	TβR3X1	Sturnira hondurensis
XP_025893663.1	TβR3	Nothoprocta perdicaria
XP_005238531.1	TβR3X1	Falco peregrinus
XP_040466106.1	TβR3X2	Falco naumanni
PIK46054.1	**putative BMPR2**	**Apostichopus japonicus**
XP_033099609.1	BMPR2	Anneissia japonica
XP_790983.2	BMPR2	Strongylocentrotus purpuratus
XP_041459768.1	BMPR2	Lytechinus variegatus
XP_033624844.1	BMPR2	Asterias rubens
XP_038058132.1	BMPR2	Patiria miniata
XP_022088502.1	BMPR2	Acanthaster planci
PIK52453.1	**putative ACVR2AX2**	**Apostichopus japonicus**
XP_030828527.1	ACVR2A	Strongylocentrotus purpuratus
XP_041458652.1	ACVR2A	Lytechinus variegatus
XP_038079379.1	ACVR2AX1	Patiria miniata
XP_033624296.1	ACVR2A	Asterias rubens
XP_042301709.1	ACVR2AX2	Sceloporus undulatus
XP_032880604.1	ACVR2AX2	Amblyraja radiata
XP_043550051.1	ACVR2AX1	Chiloscyllium plagiosum
XP_041056629.1	ACVR2A	Carcharodon carcharias
XP_022103314.1	ACVR1X4	Acanthaster planci
XP_033113857.1	ACVR1X4	Anneissia japonica
PIK59495.1	**putative ACVR1**	**Apostichopus japonicus**
NXL14502.1	ACVR1	Setophaga kirtlandii
NWI57693.1	ACVR1	Calyptomena viridis
NXY49840.1	ACVR1	Ceuthmochares aereus

(Continued)

TABLE 2 Continued

Accession no.	Protein name	Species
NXB24442.1	ACVR1	Rhagologus leucostigma
NP_001383423.1	ACVR1	Gallus gallus
KFP78435.1	ACVR1	Apaloderma vittatum
XP_032435030.1	BMPR1BX3	Xiphophorus hellerii
XP_005795012.2	BMPR1B	Xiphophorus maculatus
XP_027890208.1	BMPR1BX3	Xiphophorus couchianus
PIK51009.1	**putative BMPR1B**	**Apostichopus japonicus**
XP_797469.4	BMPR1B	Strongylocentrotus purpuratus
XP_041485680.1	BMPR1B	Lytechinus variegatus
XP_033644308.1	BMPR1B	Asterias rubens
XP_038060789.1	BMPR1B	Patiria miniata
XP_022089444.1	BMPR1B	Acanthaster planci

TABLE 3 Smad protein sequences included in the present study.

Accession no.	full name	Species
XP_041459990.1	Smad4X1	Lytechinus variegatus
XP_030827838.1	Smad4X1	Strongylocentrotus purpuratus
XP_033109387.1	Smad4X2	Anneissia japonica
XP_033646664.1	Smad4X1	Asterias rubens
XP_022081844.1	Smad4X1	Acanthaster planci
XP_038058605.1	Smad4X1	Patiria miniata
XP_032649614.1	Smad4	Chelonoidis abingdonii
XP_035384718.1	Smad4	Electrophorus electricus
XP_030421567.1	Smad4X1	Gopherus evgoodei
XP_035314530.1	Smad4X1	Cricetulus griseus
XP_016004267.1	Smad4X1	Rousettus aegyptiacus
XP_030885493.1	Smad4	Leptonychotes weddellii
XP_033127281.1	Smad6	Anneissia japonica
ADW95340.1	Smad6	Paracentrotus lividus
XP_798238.2	Smad6	Strongylocentrotus purpuratus
XP_022083936.1	Smad6	Acanthaster planci
XP_038077875.1	Smad6	Patiria miniata
XP_026878186.2	Smad6b	Electrophorus electricus
XP_015996485.2	Smad6X1	Rousettus aegyptiacus
XP_003500538.2	Smad6X1	Cricetulus griseus
XP_032621963.1	Smad6	Chelonoidis abingdonii
XP_030434864.1	Smad6	Gopherus evgoodei
XP_016003943.1	Smad7X1	Rousettus aegyptiacus
XP_007644509.3	Smad7X1	Cricetulus griseus
XP_032637417.1	Smad7X1	Chelonoidis abingdonii

(Continued)

TABLE 3 Continued

Accession no.	full name	Species
XP_030423387.1	Smad7X1	Gopherus evgoodei
XP_033124235.1	Smad3	Anneissia japonica
XP_041460773.1	Smad3	Lytechinus variegatus
XP_033624249.1	Smad3X1	Asterias rubens
XP_022083075.1	Smad3X2	Acanthaster planci
XP_038076479.1	Smad3X1	Patiria miniata
XP_006749953.1	Smad3	Leptonychotes weddellii
XP_026860560.1	Smad3a	Electrophorus electricus
XP_015996478.1	Smad3	Rousettus aegyptiacus
XP_007639432.2	Smad3X1	Cricetulus griseus
XP_028706267.1	Smad3X1	Macaca mulatta
XP_030434068.1	Smad3X1	Gopherus evgoodei
XP_032620241.1	Smad3	Chelonoidis abingdonii
XP_035391548.1	Smad2X1	Electrophorus electricus
XP_032654336.1	Smad2X1	Chelonoidis abingdonii
XP_003501086.1	Smad2X1	Cricetulus griseus
XP_006738743.2	Smad2	Leptonychotes weddellii
XP_028693529.1	Smad2X2	Macaca mulatta
XP_036085916.1	Smad2X1	Rousettus aegyptiacus
PIK47643.1	**mothers against decapentaplegic-like protein 1**	**Apostichopus japonicus**
XP_032620791.1	Smad1X2	Chelonoidis abingdonii
XP_030420831.1	Smad1X2	Gopherus evgoodei
XP_036089426.1	Smad1X1	Rousettus aegyptiacus
XP_030895811.1	Smad1	Leptonychotes weddellii
XP_026859416.1	Smad5	Electrophorus electricus
XP_032631983.1	Smad5	Chelonoidis abingdonii
XP_030428443.1	Smad5	Gopherus evgoodei
XP_003503786.1	Smad5	Cricetulus griseus
XP_014996357.1	Smad5X1	Macaca mulatta
XP_015993196.1	Smad5	Rousettus aegyptiacus
XP_022079499.1	Smad5X2	Acanthaster planci
XP_033632185.1	Smad5	Asterias rubens
XP_038055290.1	Smad5	Patiria miniata
XP_041455371.1	Smad5	Lytechinus variegatus
XP_030836187.1	Smad5	Strongylocentrotus purpuratus
ACU12852.1	Smad1	Paracentrotus lividus
PIK47644.1	**Smad1**	**Apostichopus japonicus**

FIGURE 1
Green, purple and white *A. japonicus*.

TABLE 4 Oligonucleotide primers for *A. japonicus*.

Gene	Accession no.	Primer sequence	
BMP2/4	AB057451.1	Forward	5'- CCAAAAGGCAGAAAAGCA -3'
		Reverse	5'- ACCCACAATGGCAAAGTC -3'
ACVR2A	BSL78_25911	Forward	5'- ACAGAGAAGCGTGGTGAAG -3'
		Reverse	5'- GGTAGTCATAGAGGGAGCCA -3'
Smad1	BSL78_15508	Forward	5'- ATTCTCCTTTACCAGTCCAGTT -3'
		Reverse	5'- AGCCTTCTCCAGTTCTTCC -3'
TβR2	BSL78_06251	Forward	5'- GAGCCGAAAGAAGACAGAAC -3'
		Reverse	5'- TATCGTAGAGGGAAGGACTCA -3'
Smad 2/3	BSL78_11878	Forward	5'- GCTACCGCCTCCATCTTT -3'
		Reverse	5'- CCTCCATACTGTTGTCATTGG -3'
grb2	C112121_gl_il	Forward	5'- ATCTTTCACATATTGCGAGCCAG -3'
		Reverse	5'- ATGACCATTCCGATGCCCTAA -3'

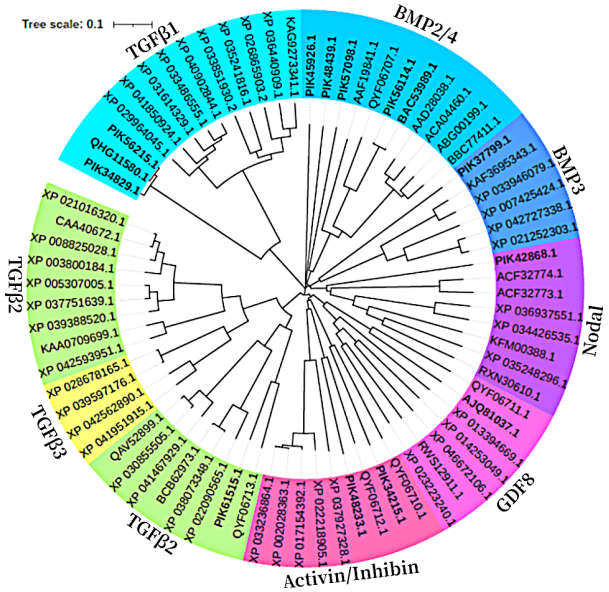

FIGURE 2
Phylogenetic analysis of the 14 ligands proteins compared to other species.

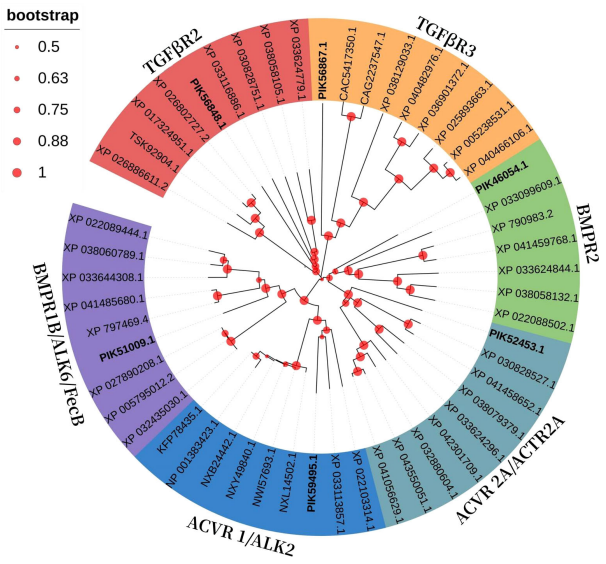

FIGURE 3
Phylogenetic analysis of the 6 receptors proteins compared to other species.

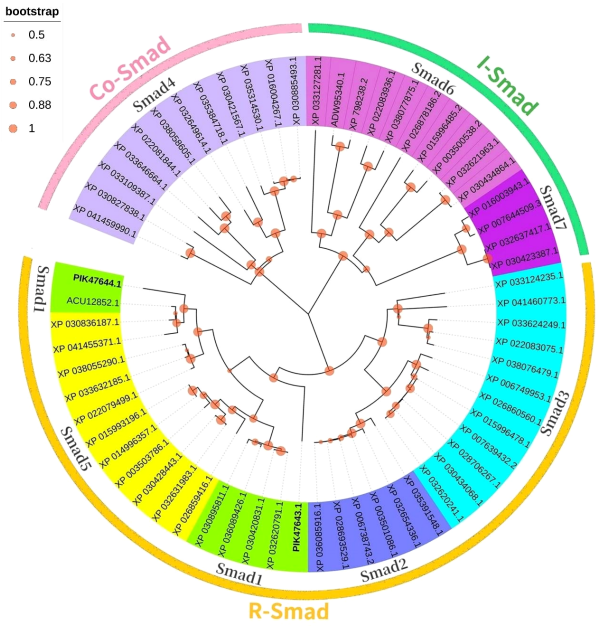

FIGURE 4
Phylogenetic analysis of the 2 Smads proteins compared to other species.

receptor type 1B, alternative name ALK6, FecB (Li et al., 2021)], BMPR2 (bone morphogenetic protein receptor type 2), ACVR1 [activin receptor type 1, alternative name ACTR1, ALK2 (Lee et al., 2017)], and ACVR2A [activin receptor type 2A, alternative name ACTR2A (Bondulich et al., 2017)].

3.3 Phylogenetic analysis of Smads

In a phylogenetic tree based on amino acid sequences of multiple Smads, the each type of Smad assembled in a cluster. The phylogenetic tree is shown in Figure 4 and the corresponding sequences are shown in Table 3. According to the phylogenetic tree, two kinds of known R-Smads from *A. japonicus* were classified into one class, the Smad1 class. Notably, they were closely related to Smad5.

3.4 mRNA levels of TGFβ signaling pathway genes in *A. japonicus* color morphs

Three different color morphs of *A. japonicus* are shown in Figure 1. mRNA levels of TGFβ signaling pathway genes in *A. japonicus* with different colors are shown in Figure 5. Compared to levels in green *A. japonicus*, the mRNA expression levels of all TGFβ signaling pathway genes were much higher in purple *A. japonicus*, with significant differences in TGFβR2 and Smad2/3 levels between morphs ($p < 0.05$). The mRNA expression levels of BMP2/4, ACVR2A, Smad1, and TGFβR2 of the purple individuals were significantly lower than those in the white morph ($p < 0.05$), with no difference in Smad 2/3 ($p > 0.05$).

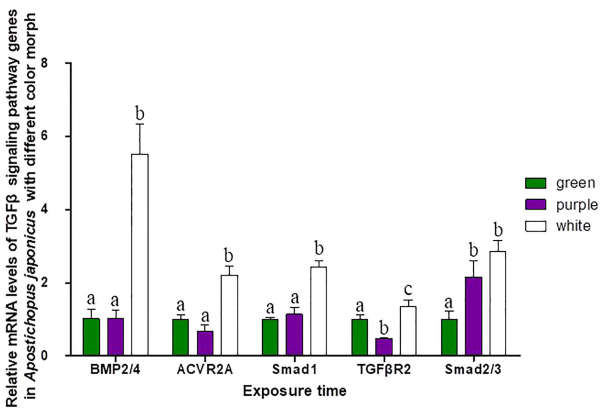

FIGURE 5
Relative mRNA levels of TGFβ signaling pathway genes in *A. japonicus* of different body colors. Means followed by different lower-case letters are significantly different at $P<0.05$.

3.5 mRNA levels of TGFβ signaling pathway genes during pigmentation in *A. japonicus*

The pigmentation process in purple sea cucumber was divided into four stages: A, B, C, and D (Figure 6). mRNA levels of TGFβ signaling pathway genes at each stage are shown in Figure 7. BMP2/4 and Smad2/3 levels did not differ among pigmentation stages in *A. japonicus* ($p > 0.05$). Compared to levels at stage A, the mRNA expression of ACVR2A was lower at stage C and higher at stage D. The mRNA expression levels of Smad1 and TGFβR2 were significantly higher at stages B and C than at stage A. As time progressed, the expression level of TGFβR2 began to decrease, with lower levels at stage D than at stages B and C.

4 Discussion

The TGFβ superfamily consists of over 50 structurally related ligands and can be divided into two subfamilies based on sequence similarity and the specific signaling pathways they activate: the TGFβ/activin/Nodal subfamily and BMP/GDF/AMH (anti-Mullerian hormone) subfamily (Shi and Massagué, 2003; Massagué, 2012; Miyazono et al., 2018). These have been described in a large number of studies of TGFβ superfamily ligand, receptor, and R-Smad interactions in various species (Piek et al., 1999; Attisano and Wrana, 2002; de Caestecker, 2004; Schilling et al., 2008; Romano et al., 2012) and were detected in the sea cucumber genome (Tables 1–3) . Ligand–receptor–R-Smad interactions in *A. japonicus* were inferred, as shown in Table 5, and putative TGFβ-mediated signaling pathways in *A. japonicus* are shown in Graphical Abstract. In the first subfamily, ligands (TGFβ1, TGFβ2, Activin B, Inhibin, and Nodal), Receptor II (TGFβR2 and ACVR2A/ACTR2A), and R-Smads (Smad2, 3) were found in *A. japonicus*. In the second subfamily, ligands (BMP2, BMP4, BMP3, and BMP3B), Receptor I (BMPR1B/ALK6), Receptor II (ACVR2A/ACTR2A and BMPR2), and R-Smad (Smad1, 5 and Smad2, 3) were found in *A. japonicus*.

The TGFβ signaling pathway is considered a good marker for the evolution of animal genomes (Long, 2019). Three TGFβ isoforms are known in mammals (Derynck et al., 1985; Van Obberghen-Schilling et al., 1987; ten Dijke et al., 1988; Miller et al., 1989a; Miller et al., 1989b) and in birds (TGFβ2, β3, and β4) (Jakowlew et al., 1988a; Jakowlew et al., 1988c; Jakowlew et al., 1988b; Jakowlew et al., 1990), two in amphibians (TGFβ2 and TGFβ5) (Kondaiah et al., 1990; Rebbert et al., 1990), and four in fish (TGFβ1, β2, β3 and β6) (Funkenstein et al., 2010). In the

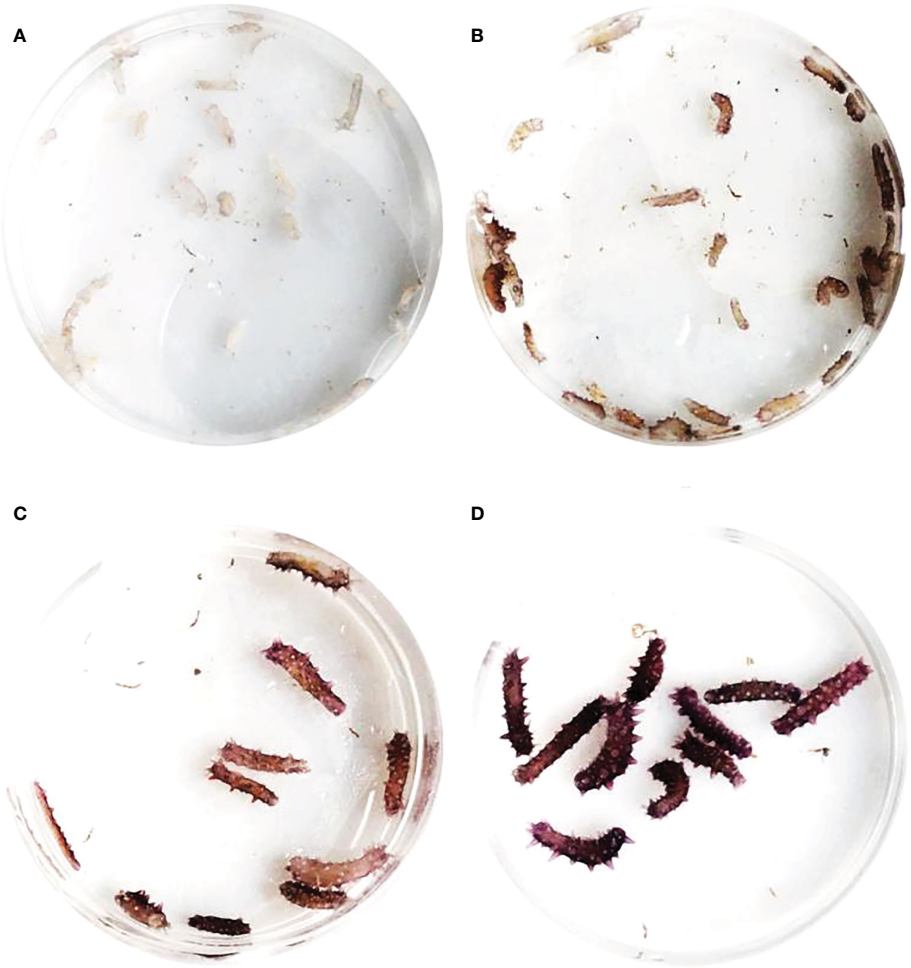

FIGURE 6
Pigmentation stages of purple *A. japonicus*.

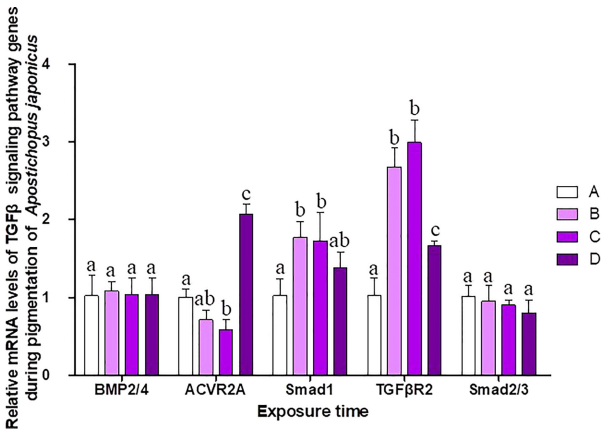

FIGURE 7
Relative mRNA levels of TGFβ signaling pathway genes during different pigmentation stages of purple *A. japonicus*. Means followed by different lowercase letters are significantly different at $P<0.05$.

present study, two TGFβ ligand isoforms (TGFβ1 and TGFβ2) were identified in *A. japonicus* (Figure 2). A BLAST search against GenBank entries (putative TGFβ1 like, PIK34829.1, putative TGFβ1X1, PIK56215.1, and putative TGFβ1X1, QHG11580.1) revealed high amino acid sequence homology with TGFβ1 (Figure 2 and Table 1). It is worth noting that although TGFβ2 was classified as TGFβ2, it was closely related to TGFβ3 (Figure 2 and Table 1). Activin is the dimer of β-subunits, activin A (β_A-β_A), activin B (β_B-β_B), and activin AB (β_A-β_B). Inhibin A, B, C are dimers composed of an α-subunit associated with β_A, β_B, and β_C (Burger, 1988; Mellor et al., 2000; Ushiro et al., 2006). Accordingly, the putative activin BX1 and putative inhibin beta C chain-like of *A. japonicus* clustered in the Activin/Inhibin cluster on the phylogenetic tree and showed a relatively low identity (Figure 2 and Table 1). The TGFβ family member nodal of *A. japonicus* were assigned to the Nodal cluster (Figure 2 and Table 1). In summary, sea cucumber possessed the complete TGFβ/activin/Nodal ligand subfamily.

Although no typical receptor I was found in the TGFβ/activin/Nodal subfamily (Table 5), significant differences in the mRNA expression levels of TGFβR2, ACVR2A, and Smad2/3 were detected in sea cucumbers with different body colors (Figure 5). Expression levels of TGFβR2 during different pigmentation stages of purple *A. japonicus* were significantly higher than those during the unpigmented period; however, the expression levels of Smad2/3 did not differ significantly ($p > 0.05$) (Figure 7). This indicates that TGFβR2 is involved in the regulation of the coloration process of *A. japonicus*; however, its specific regulatory mechanism is still unclear. TGFβR2 and Smad2/3 also differ significantly between peripheral blood lymphocytes of patients with systemic lupus erythematosus and a normal control group (Sun et al., 2013). When ACVR2A function is reduced in melanocytes, gray hair develops (Han et al., 2012). These findings are consistent with the higher expression of ACVR2A in the white morph than in the other color morphs of *A. japonicus*. More broadly, there are ethnic differences in TGFβ signaling in African American and Caucasian skin (Fantasia et al., 2013). Taken together, these studies support the hypothesis that the TGFβ/activin/Nodal subfamily is involved in the regulation of body color of *A. japonicas*.

The second subfamily involved BMP/GDF/AMH. BMP is the largest subfamily of TGFβ ligands. In the current study, two BMPs, BMP2/4 and BMP3, were found. The BMPs (PIK57098.1) of *A. japonicus* were classified as BMP2/4. Sj-BMP2/4 was recorded in GenBank with two different accession numbers (PIK56114.1 and BAC53989.1). A Blast

TABLE 5 TGFβ superfamily ligand-receptor-Smad specificity.

Subfamily	Ligand	Receptor I	Receptor II	R-Smad
TGFβ/activin/Nodal	TGFβ1	no records	TGFβR2	Smad2, 3
	TGFβ2	no records	TGFβR2	Smad2, 3
	Activin B	no records	ACVR2A/ACTR2A	Smad2, 3
	Inhibin	No type I receptor	ACVR2A	no specific R-Smads
	Nodal	no records	ACVR2A/ACTR2A	Smad2, 3
BMP/GDF/AMH	BMP2	BMPR1B/ALK6	ACVR2A/ACTR2A and BMPR2	Smad1, 5
	BMP4	BMPR1B/ALK6	ACVR2A/ACTR2A and BMPR2	Smad1, 5
	BMP3	No type I receptor	no records	no records
	BMP3B	no records	ACVR2A/ACTR2A	Smad2, 3

analysis showed that the two proteins were highly homologous (query cover: 100%, identity: 99.29%). *A. japonicus* and *Stichopus japonicus* are two different names for the same species (Chang et al., 2009). Accordingly, Sj-BMP2/4 corresponds to BMP2/4 of *A. japonicus*. In this study, the mRNA expression of BMP2/4 did not differ among pigmentation stages of purple *A. japonicus* (Figure 7). However, BMP2/4 expression was significantly higher in the white morph than in the green and purple morphs (Figure 5), suggesting that BMP2/4 is closely related to formation of the white body color. Numerous studies have shown that BMP2 and BMP4 can induce stem cells to differentiate into adipocytes and to differentiate into white adipocytes (Ahrens et al., 1993; Wang et al., 1993; Sottile and Seuwen, 2000; Bowers and Lane, 2007; Gomes et al., 2012). White sea cucumbers are uniformly white on the dorsal and ventral sides, while purple and green sea cucumbers have obvious color differences, i.e., the dorsal side is darker than the ventral side (Figures 1, 6). The specificity of BMP2/4 was receptor I (BMPR1B)–receptor II (ACVR2A and BMPR2)–R-Smad (Smad1,5). In *A. japonicus*, their expression levels in white sea cucumber were significantly higher than those in the purple and green sea cucumbers (Figure 5). These results suggest that the BMP2/4-induced differentiation of white adipocytes in *A. japonicus* is regulated by this signaling pathway. Functional tests, including gain- or loss-of-function assays, using exogenous BMPs or BMP antagonists are necessary to validate the roles of this pathway in *A. japonicus*. In the GDF gene family, only GDF8 was detected in *A. japonicus* (Figure 2 and Table 1). There was no record of AMH in sea cucumber. Accordingly, the BMP/GDF/AMH ligand subfamily in sea cucumber is incomplete.

5 Conclusions

In summary, 14 TGFβ signaling pathway members were identified in *A. japonicus* for the first time, including 7 ligands, 6 receptors, and 1 R-Smad. Detailed phylogenetic and gene expression analyses support the hypothesis (Graphical Abstract) that both subfamilies of the TGFβ superfamily were involved in the regulation of pigmentation in different color morphs of *A. japonicus*. The TGFβ/activin/Nodal subfamily was complete and contributed to the regulation of different color morphs. The BMP/GDF/AMH subfamily was incomplete, and the BMP2/4-induced differentiation of white adipocytes was regulated by the BMP2/4–ACVR2A–Smad1 signaling pathway.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Author contributions

LY and CL conceived and designed the experiments. Material preparation, data collection and analysis were performed by LY, BZ, QW, XJ, SH, WH. The first draft of the manuscript was written by LY and all authors commented on previous versions of the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

Author XJ was employed by the company Country Conson CSSC Qingdao Ocean Technology CO., Ltd.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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