Biotechnology Reports 19 (2018) e00262

Contents lists available at ScienceDirect

Biotechnology Reports

journal homepage: www.elsevier.com/locate/btre

Molecular cloning and characteristics analysis of *Pmtgfbr1* from *Pinctada fucata martensii*

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ARTICLE INFO

Article history:
Received 15 March 2018
Received in revised form 23 May 2018
Accepted 3 June 2018

Keywords:
Pmtgfbr1
Growth traits
Correlation analysis
Pinctada fucata martensii

ABSTRACT

Pinctada fucata martensii is cultured for pearl production. Growth improvement has received considerable research interest. Transforming growth factor β type I receptor (TβR-I), which is involved in signals transmission of transforming growth factor beta (TGF-β), participates in cell proliferation and growth. In this study, we characterized a *Tgfbr1* gene which encoded TβR-I from *P. fucata martensii* (*Pmtgfbr1*). *Pmtgfbr1* cDNA contains an open reading frame of 1569 bp and encodes a polypeptide of 522 amino acids (aa). Pmtgfbr1 possesses a typical TβR-I structure (extracellular receptor ligand domain, transmembrane domain, and cytoplasmic tyrosine kinase catalytic domain). *Pmtgfbr1* is expressed in all the studied tissues and exhibited the highest expression level in the adductor muscle. Moreover, *Pmtgfbr1* exhibited the lower expression level in the larger group (L) than that in the smaller group (S) and is negatively correlated with growth traits ($P < 0.01$). Our results indicated that *Pmtgfbr1* is a candidate functional gene associated with growth traits.

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1. Introduction

The transforming growth factor β(TGF-β) superfamily comprises bone morphogenetic proteins, activins, TGF-βs, and other related factors [1,2]. It has attracted considerable research attention because of the abilities of its members to regulate cell migration, adhesion, proliferation, differentiation, and death throughout the entire lifespan of an organism [3–5]. TGF-β family members transmit signals through signaling systems that involve types I and II serine/threonine kinase receptors [6–8]. In receptor activation, the TGF-β type I receptor (TβR-I) mainly acts downstream of the TGF-β type I receptor (TβR-II) and sometimes determines the specificity of intracellular signals [9,10].

Given the importance of TGF-β signaling for cell bioprocesses [1], it is a potential target of strategies for the control of human cancer progression, suppression of tumors, and regulation of animal growth [11–13]. TβR-I, TβR-II, and Smad protein genes have been widely studied in numerous species [14–16] especially in the oysters [17–21] which represents the existence of TGF-β pathway in bivalves. Polymorphic TGF-β receptors from *Crassostrea gigas* could serve as markers for genes associated with fast growth and be applied in oyster breeding [22]. In *Zhikong scallop*, Tgfbr1 negatively regulates growth and may thus be used as a candidate marker for marker-assisted breeding of this species [23].

Pinctada fucata martensii (synonymous to *P. fucata* and *P. martensii*) as an important species for pearl culture is widely studied for its biomineralization and immune system for the pearl production purpose [24,25]. The TGF-β signal and receptor genes of the pearl oyster *P. fucata martensii* have been also analyzed [26,27]. Most studies showed that TGF-β signal pathway is associated with biomineralization in *P. fucata martensii*. However, studies on the pearl oyster growth remain relatively limited which is also a crucial factor for the pearl culture. Therefore, in this study, we cloned *Pmtgfbr1*, the TβR-I gene of *P. fucata martensii* and estimated the relationship between *Pmtgfbr1* and growth traits of pearl oysters.

2. Methods and materials

2.1. Animals and sample collection

Pearl oysters *P. fucata martensii* were obtained from the Breeding Base, Xuwen, Zhanjiang, Guangdong Province, China (20°250 N, 109°570 E). The marginal zone of the mantle (ME),

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https://doi.org/10.1016/j.btre.2018.e00262
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central zone of the mantle (MC), adductor muscle (A), gill (GI), hepatopancreas (HE), and hemocytes (B) were obtained from adult pearl oysters.

2.2. RNA extraction and cDNA synthesis

Total RNA was extracted from various tissues of 8 adult pearl oysters using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and RNA quality was determined with 1.0% agarose gel electrophoresis and NanoDrop ND1000 spectrophotometer (Thermo scientific, Waltham, MA, USA). Reverse transcription with M-MLV reverse transcriptase (Promega, Madison, WI, USA) was performed with total RNA as template.

2.3. Full-length Pmtgfbr1

The partial sequence of the *Pmtgfbr1* gene was obtained from the genomic data of *P. fucata martensii* [24]. RACE reactions were performed with SMART RACE cDNA Amplification Kit (Clontech, USA) and template cDNA from total RNA obtained from mantle tissue. Specific amplification products were obtained through nested polymerase chain reaction (PCR). Primers involved in this study were designed in accordance with the partial sequence of *Pmtgfbr1* and are shown in Table 1.

2.4. Sequence and phylogenetic analysis

PCR products containing the 5′-UTR and 3′-UTR were sequenced, and the full length of *Pmtgfbr1* was obtained with DNAMAN software. The open reading frame (ORF) of *Pmtgfbr1* was identified with ORF finder (http://www.ncbi.nlm.nih.gov/gorf/orfig.cgi). Multiple-sequence alignments were generated with protein sequences from other species by using ClustalX (http://www.ebi.ac.uk/Tools/msa/clustalo/). Protein domain was predicted using SMART (http://smart.embl-heidelberg.de/). The intracellular region of *Pmtgfbr1* was submitted to Phyre2 online at http://www.sbg.bio.ic.ac.uk/phyre2/protocol for three-dimensional model construction. Chimera 1.8.1 was used to display the model. A phylogenetic tree was constructed by neighbor-joining (NJ) method with MEGA version 6.1 and tested for reliability over 1000 bootstrap replicates.

2.5. Pmtgfbr1 expression pattern in adult tissues

Real-time quantitative PCR (qRT-PCR) analysis was performed with Thermo Scientific DyNAmo Flash SYBR Green qPCR Kit (Thermo Scientific) in Applied Biosystems 7500/7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, CA, USA) to identify the expression pattern of *Pmtfgbr1*. Table 1 presents the specific primers used in this analysis. Transcripts were relatively quantified through $2^{-\Delta CT}$ method with GAPDH as the internal control [28,29].

Table 1
Primes used in the study.

Primes	Sequence (5′-3′)	application
5′-outer	GTCTAAGCATCACGGTCTGGTAAATCTC	Outer PCR
5′-inner	TTTACTGCTACGCTTTCTGCTCGCC	Inner PCR
3′-outer	TGTTGACCTCGCTCCATCAGACAGAGT	Outer PCR
3′-inner	GAGAGGCGATGTCTACTCGTTTGGTTTG	Inner PCR
Pmtgfbr1-A	TAGGCGAAAAACGCAACGAT	qRT-PCR
Pmtgfbr1-S	AACTCCGACCTTGGCACCCC	qRT-PCR
GAPDH-A	CGTTGATTATCTTGGCGAGTG	qRT-PCR
GAPDH-S	GCAGATGGTGCCGAGTATGT	qRT-PCR

2.6. Pmtgfbr1 expression in two sized groups

The samples were obtained from the base stock in our breeding program. 260 pearl oysters (two years old) were randomly collected from the base stock and sized according to shell length measurement [30]. 20 samples with larger shell length (L) and 20 samples with smaller shell length (S) were used for gene expression analysis. Growth traits of two sized groups were shown in Table S1. The adductor muscle was dissected and stored in liquid nitrogen. The expression levels of *Pmtgfbr1* in the two groups (L and S) were detected using the above method and were relatively quantified by $2^{-\Delta CT}$ method with GAPDH as the internal control.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the differences in mean *Pmtgfbr1* expression levels among different tissues. *Pmtgfbr1* expression levels in the L and S groups were compared using *t*-test. The significance level for the analyses was set at $P < 0.05$. Correlations among *Pmtgfbr1* gene expression levels and growth traits were estimated using the Pearson method. All analyses were performed with SPSS 19.0 software.

3. Results

3.1. Cloning and sequencing analysis of Pmtgfbr1

The full-length sequence of *Pmtgfbr1* (2210 bp) contained a 45-bp 5′-UTR and a 596-bp 3′-UTR with a 39-bp poly-(A) tail. The sequence analysis of *Pmtgfbr1* showed that it contained an ORF of 1569 bp and encoded 522 amino acids (aa). The deduced aa sequence of *Pmtgfbr1* had a typical activating receptor, a signal peptide, a transmembrane domain, a GS motif, and a serine/threonine protein kinase domain (Fig. 1a). The sequence analysis of *Pmtgfbr1* showed that the signal peptide was 1–22 aa in length, and the transmembrane domain was 134–156 aa in length (Fig. 1b). The *Pmtgfbr1* gene and deduced protein sequences were deposited in the GenBank database under the accession number AIA98699.1.

3.2. Homology analysis of Pmtgfbr1

The deduced aa sequence of Pmtgfbr1 was homologous to that of Tgfbr1. The homology analysis of Pmtgfbr1 was performed with Clustal X2 software. Pmtgfbr1 was compared with Tgfbr1 from *Crassostrea gigas* (EKC41469.1), *Mizuhopecten yessoensis* (XP_021365929.1), *Azumapecten farreri* (AFQ23184.1), and *Lingula anatine* (XP_013382711.1). The results indicated that Pmtgfbr1 had high homology with other Tgfbr1 (Fig. 2). Pmtgfbr1 showed a completely conserved L45 loop. Furthermore, Pmtgfbr1 shared the highest identity (73%) with Tgfbr1 from *C. gigas* (EKC41469.1) and followed by with those from *A. farreri* (66%), *M. yessoensis* (65%), and *L. anatine* (63%).

3.3. Three-dimensional model analysis of Pmtgfbr1

The three-dimensional model analysis of the Pmtgfbr1 kinase domain showed that its tertiary structure is similar to the kinase domain of Tgfbr1 from *Chlamys farreri* (Fig. 3), in which the receptor uses the L45 loop to interact with smad proteins [7]. This result indicated that Pmtgfbr1 functions similarly to Tgfbr1 from other animals.

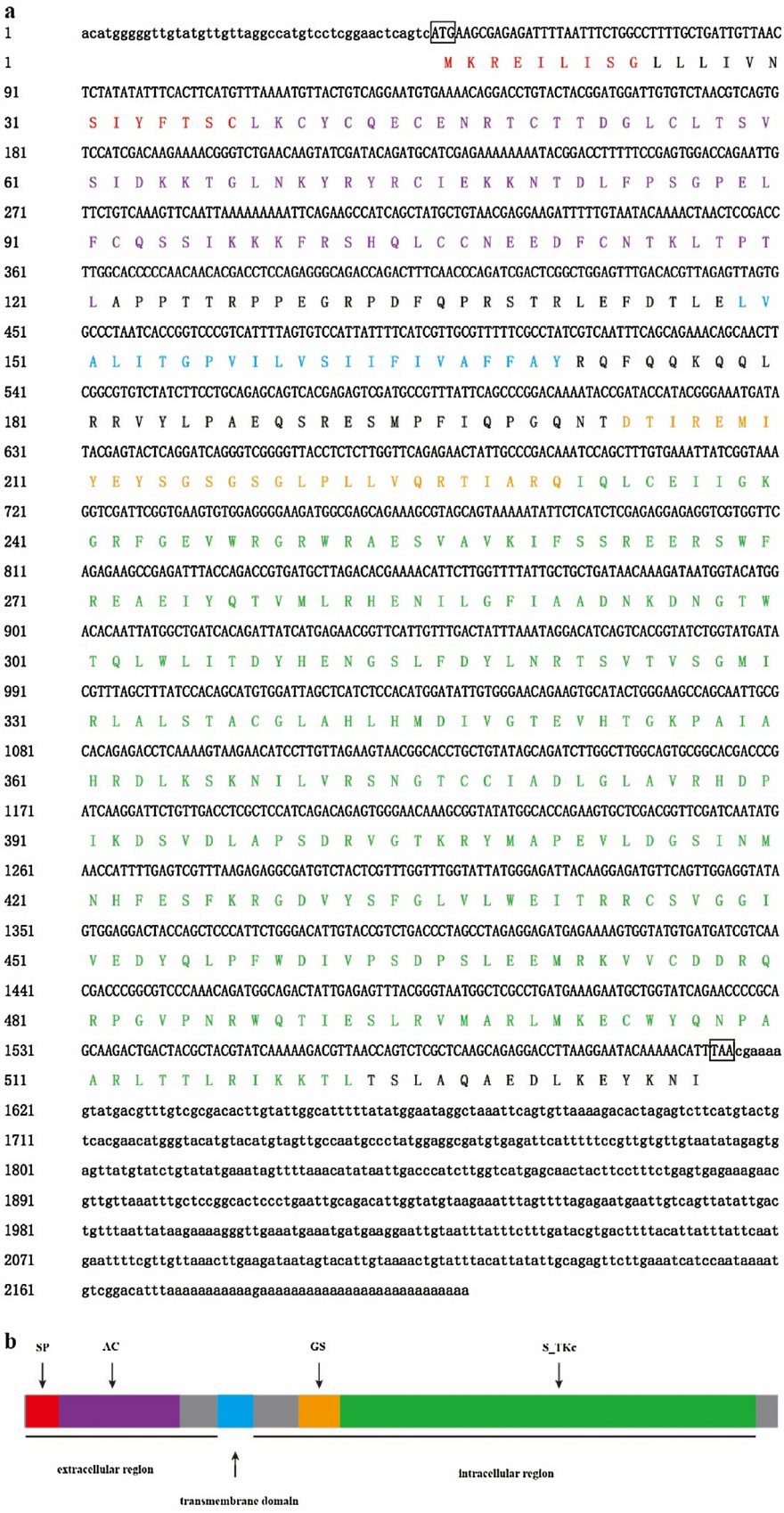

Fig. 1. Full-length cDNA and amino-acid (aa) sequence analysis of *Pmtgfbr1* from *P. fucata martensii*. (a) Full-length cDNA of *Pmtgfbr1*. Numbers on the left represent nucleotide and amino acid positions. The 5′ and 3′ UTRs are indicated by small letters. The open reading frame and the deduced aa sequences are indicated by capital letters. The initiation codon (ATG) and stop codon (TAA) are represented by nucleotides surrounded by frames. Sequences in purple and green represent the activin_recp domain and S_TKc domain, respectively. Sequences in red and blue indicate the signal peptide and the transmembrane domain, respectively. The GS motif is in orange. (b) aa sequence analysis of *Pmtgfbr1*. SP: signal peptide, AC: activin_recp, GS: GS motif, S_TKc: serine/threonine protein kinases.

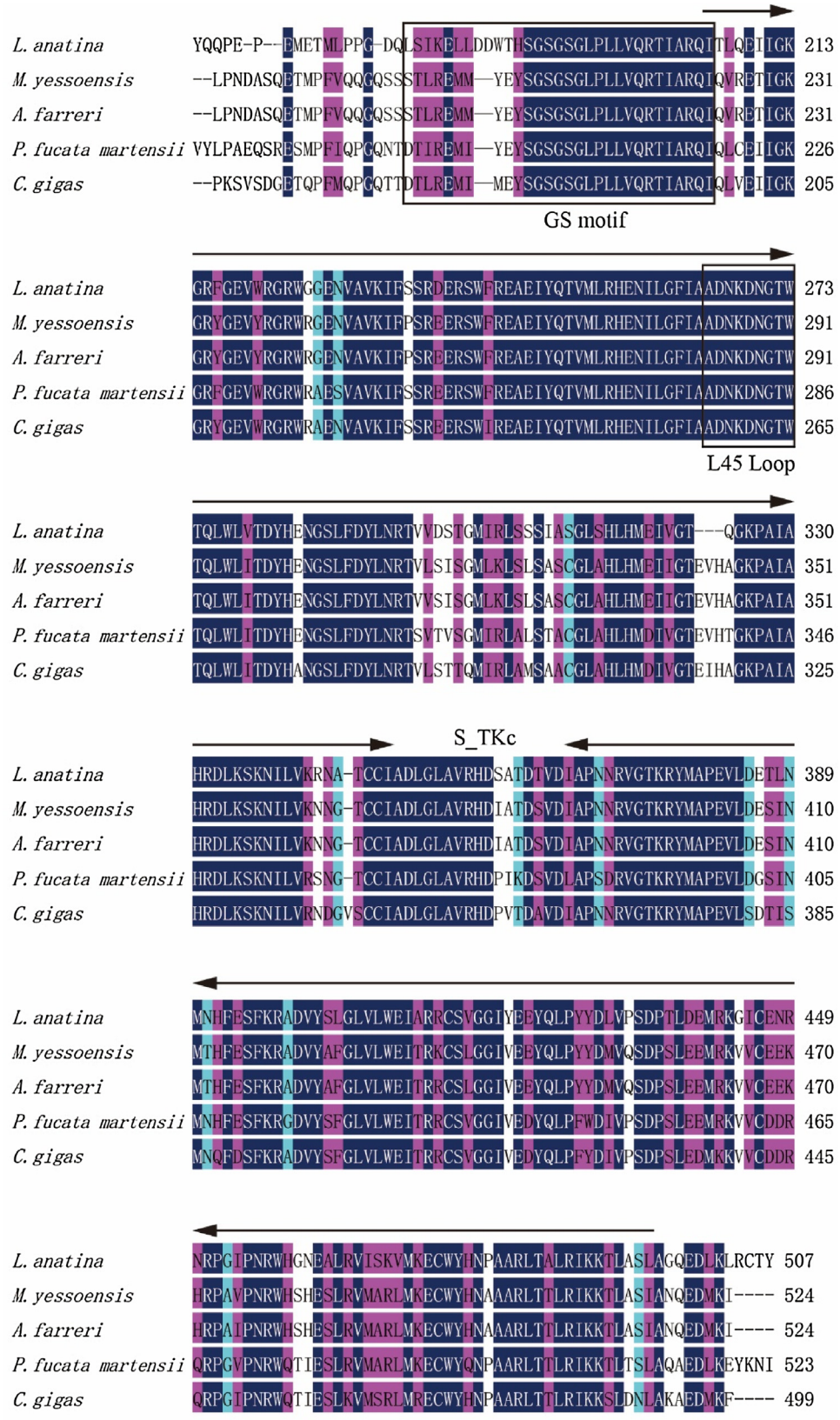

Fig. 2. Multiple-sequence alignment of Pmtgfbr1 aa sequences. Conserved aa sequences are indicated by a dark blue background. Highly similar aa sequences are indicated by a pink background. Weakly similar aa sequences are indicated by a light blue background. Numbers on the right show the position of the aa sequence alignment. The accession numbers of the sequences used in this alignment are as follows: *P. fucata martensii* (AIA98699.1), *Crassostrea gigas* (EKC41469.1), *Mizuhopecten yessoensis* (XP_021365929.1), *Azumapecten farreri* (AFQ23184.1), *Lingula anatine* (XP_013382711.1). S_TKc represents serine/threonine protein kinases. Sequences surrounded by frames represent the GS motif and L45 loop.

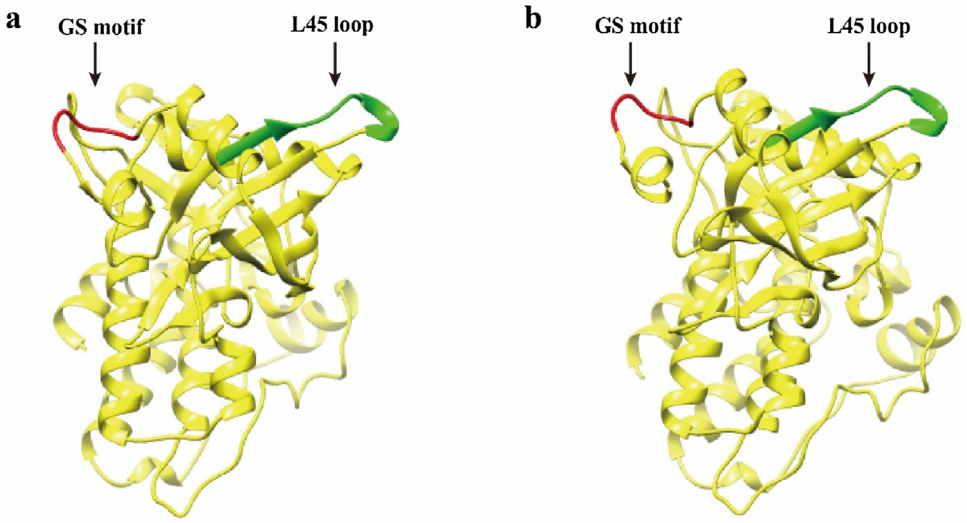

Fig. 3. Molecular model of the three-dimensional structure of the kinase domain of Pmtgfbr1 (a) and *Chlamys farreri* Tgfbr1 (b). The GS motif and L45 loop are colored red and green, respectively. https://doi.org/doi:10.1371/journal.pone.0051005.

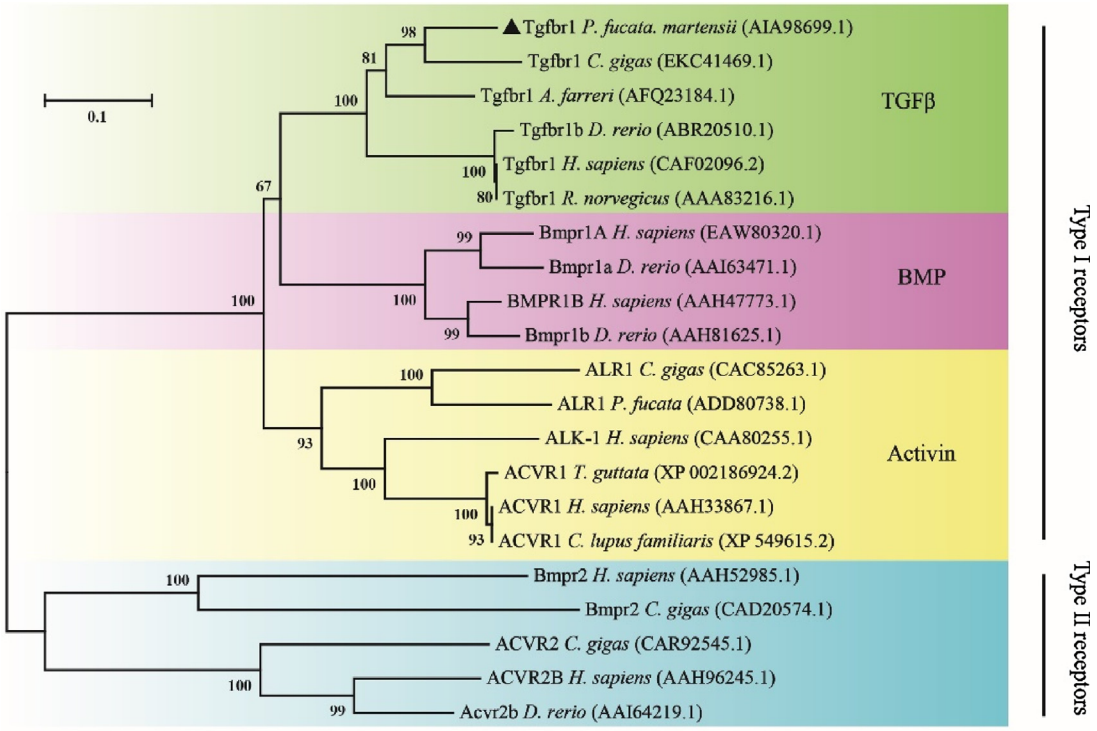

Fig. 4. Phylogenetic tree of Pmtgfbr1 and other TGF-β superfamily receptors. The phylogenetic tree was constructed using MEGA software 6.05 through the neighbor-joining method with 1000 bootstrap replications. Numbers at the forks indicate bootstrap proportions. The scale bar indicates a branch length of 0.1. The protein sequences used for phylogenetic analysis include the following: *P. fucata martensii* tgfbr1 (AIA98699.1), *Azumapecten farreri* TGF-beta type 1 receptor (AFQ23184.1), *Homo sapiens* transforming growth factor receptor beta 1 (CAF02096.2), *Rattus norvegicus* transforming growth factor beta type I receptor (AAA83216.1), *Danio rerio* transforming growth factor-beta receptor type Ib (ABR20510.1), *H. sapiens* bone morphogenetic protein receptor, type IA (EAW80320.1), *Danio rerio* Bmpr1a protein (AAI63471.1), *Homo sapiens* BMPR1B (AAH47773.1), *Danio rerio* Bone morphogenetic protein receptor, type 1b (AAH81625.1), *C. gigas* activin-like type 1 receptor (CAC85263.1), *H. sapiens* ACVR1 protein (AAH33867.1), *H. sapiens* ALK-1 (CAA80255.1), *H. sapiens* ACVR2B protein (AAH96245.1), *D. rerio* Acvr2b (AAI64219.1), *C. gigas* activin type II receptor (CAR92545.1), *H. sapiens* Bone morphogenetic protein receptor, type II (serine/threonine kinase) (AAH52985.1), *C. gigas* bone morphogenic protein type 2 receptor (CAD20574.1), *C. gigas* TGF-beta receptor type-1 (EKC41469.1), *Pinctada fucata* activin-like receptor 1-like protein (ADD80738.1), *Taeniopygia guttata* activin receptor type-1 (XP_002186924.2), *Canis lupus familiaris* activin receptor type-1 (XP_549615.2).

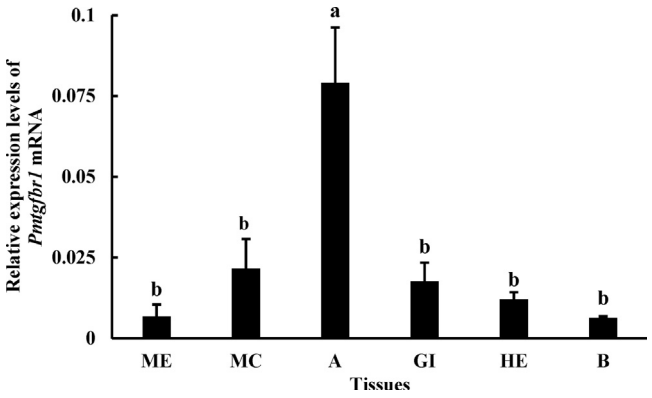

Fig. 5. *Pmtgfbr1* expression levels in different *P. fucata martensii* tissues. ME, marginal zone of mantle; MC, central zone of mantle; A, adductor muscle; GI, gill; HE, hepatopancreas; and B, hemocytes. The GAPDH gene was used as the reference gene. Different letters indicate significant differences ($P < 0.05$) determined through one-way ANOVA, and bars represent standard deviation.

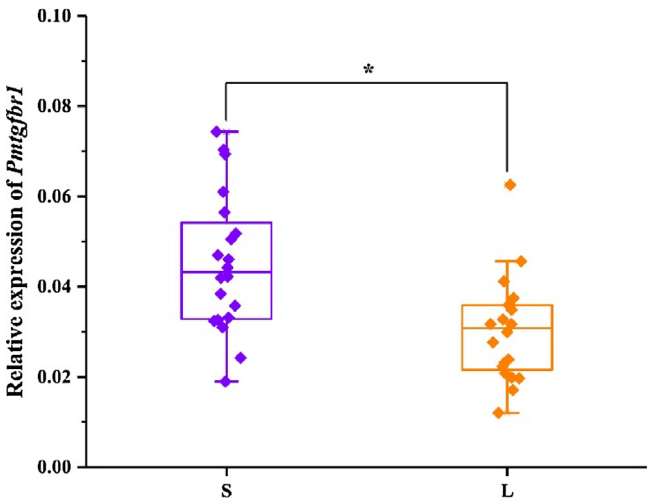

Fig. 6. *Pmtgfbr1* expression in the L and S groups. The differences in *Pmtgfbr1* gene expression levels between the S and L groups were analyzed through *t*-test. The GAPDH gene was used as the reference gene. "*" Indicates significant differences between the L and S groups ($P < 0.05$).

3.4. Construction of the Tgfbr1 phylogenetic tree

To investigate the relationship between Pmtgfbr1 and other TβR-I and TβR-II receptors, a phylogenetic tree was constructed with 21 associated sequences from various phyla by using the NJ method and 1000 bootstrap replications. Pmtgfbr1 showed a high degree of conservation with TβR-I from other phyla (Fig. 4).

3.5. Expression analysis of Pmtgfbr1

The expression pattern of *Pmtgfbr1* at different tissues was detected through qRT-PCR to further confirm the existence of *Pmtgfbr1*. *Pmtgfbr1* expressed in the ME, MC, A, GI, HE, and B of adult pearl oysters (Fig. 5). *Pmtgfbr1* expression in adductor muscle was significantly higher than that in other tissues ($P < 0.05$) . However, it was not significantly different among ME, MC, GI, HE, and B.

3.6. Pmtgfbr1 expression levels in two groups and its relationship to growth traits

The L and S groups used in the differential expression and growth characterization experiments were utilized to validate the relationship of *Pmtgfbr1* with growth of *P. fucata martensii*. qRT-PCR results indicated that *Pmtgfbr1* expression levels were significantly lower in the L group than that in the S group ($P < 0.05$) (Fig. 6). Pearson's correlation analysis between *Pmtgfbr1* expression levels and growth traits showed that *Pmtgfbr1* expression is significantly correlated with shell length ($R = -0.767$, $P < 0.01$), shell height ($R = -0.794$, $P < 0.01$), shell width ($R = -0.790$, $P < 0.01$), total weight ($R = -0.767$, $P < 0.01$), tissue weight ($R = -0.694$, $P < 0.01$), and shell weight ($R = -0.783$, $P < 0.01$) (Table 2).

4. Discussion

TGF-β signaling regulates numerous cellular bioprocesses [1], and many studies have focused on the genes involved in this signaling pathway to elucidate its potential mechanisms [31–34]. Many associated growth factors control the cell development and homeostasis of metazoans, and mutations in these pathways cause various human diseases [35–37]. Although the molecular breeding of pearl oysters with improved pearl production has received considerable attention, studies on the relationship between the genes and growth traits of pearl oysters remain limited.

By cloning and subjecting *Pmtgfbr1* to sequence analysis, we found that the deduced Pmtgfbr1 exhibits the typical features of TβR-I receptor aa sequences. The intracellular region of Pmtgfbr1 is characterized by a serine/threonine kinase domain [38,39], GS motif [40], and L45 loop, which perfectly matches the consensus motif of other Tgfbr1 proteins. The conserved serine/threonine kinase structures are crucial for determining the specificity of TβR-I for smad proteins [41]. The multiple-sequence alignment of the whole aa sequences of Pmtgfbr1 and other homologous Tgfbr1 proteins revealed that the highly conserved serine/threonine protein kinases, identical SGSGSG sequences, and L45 loop are important features for signal transmission in the TGF-β pathway and that TβR-II phosphorylates TβR-I in the Ser of the SGSGSG motif [42–44]. Therefore, Pmtgfbr1 may have similar functions as TβR-I.

Phylogenetic analysis showed that Pmtgfbr1 has a high degree of conservation with TβR-I. Moreover, comparing the three-dimensional structure of the *Pmtgfbr1* kinase domain with the kinase domain of Tgfbr1 from *C. farreri* also showed that *Pmtgfbr1* is highly conserved [23], providing additional evidence for the

Table 2
Correlation analysis between *Pmtgfbr1* expression and growth traits of *P. fucata martensii*.

		Shell length	Shell height	Shell width	Total Weight	Tissue weight	Shell weightlll
Pmtgfbr1 expression levels	*R*	−0.767**	−0.794**	−0.790**	−0.767**	−0.694**	−0.783**
	P	0.000	0.000	0.000	0.000	0.000	0.000

Note: The number in the table indicates the correlation coefficient (*R*). $R > 0$ indicates positive correlation, and $R < 0$ represents negative correlation. Correlations with "*" are statistically significant at $P < 0.05$. Correlations with "**" are statistically significant at $P < 0.01$.

potential function of this gene in Tgfbr1 activation and the interactions between Tgfbr1 and smad [45].

Pmtgfbr1 expressed in all sampled adult tissues. This expression pattern indicated the extensive existence of the TGF-β signaling pathway, which is necessary for diverse bioprocesses [46]. Adductor muscle presented the highest expression level of *Pmtgfbr1* among all tissues, indicating that *Pmtgfbr1* has potential roles in muscle growth and regulation [13]. The TGF-β signaling pathway, which transmits signals via Tgfbr1, is also expressed in the skeletal muscle of mammals, such as mice and humans, and is involved in myogenesis and muscle growth [47–50]. *Tgfbr1* genes are highly expressed in the muscle tissue of other aquatic species, such as fish, scallops, and other oysters [14,51], providing further evidence for its potential role in muscle growth. Developmental transcriptome analysis of *P. fucata martensii* showed *Pmtgfbr1* was up-regulated in the early trochophore and gastrula which indicated that it was associated in the early development of pearl oyster (Fig. S1) [24]. Therefore, Pmtgfbr1 may be a potential gene participated in the growth of early development and muscle growth.

TGF-β could inhibit gene expression specific to skeletal muscle and modulate cell proliferation [52–55]. In this study, we identified correlations between *Pmtgfbr1* expression in adductor muscle and *P. fucata martensii* growth traits. *Pmtgfbr1* expression level is significantly lower in the L group than that in the S group. The transcriptome of the TL (Transcriptome of L group) and TS (Transcriptome of S group) groups [24] also presented that *Pmtgfbr1* was up-regulated in the TS group which is consistent with the result of expression pattern in the L and S groups (Fig. S2). On the other hand, there existed significantly negative correlation between gene expression and various traits. This finding indicated that Pmtgfbr1 had a negative effect on oysters in the fast-growing group and its associated pathway are involved in pearl oyster growth. Guo et al. showed a similar correlation between striated muscle mass and Tgfbr1 expression [23]. TGF-β may be involved in inhibiting skeletal muscle differentiation and in muscle growth [49].

5. Conclusion

Pmtgfbr1 possesses the conserved domain of Tgfbr1 and is expressed in all sampled tissues. There existed negative correlation between *Pmtgfbr1* expression levels and growth traits of *P. fucata martensii*. These results indicated that *Pmtgfbr1* genes negatively affect growth of *P. fucata martensii*.

Conflicts of interest

The authors declare no conflict of interest.

Financial support

The research was financially supported by Guangdong Marine and Fishery Bureau (B201601-Z10,Z2014009 and Z2015004), Science and Technology Program of Guangdong Province (2017A030303076 and 2017A030307024), Modern Agricultural Industrial System (CARS-049) and Project of Enhancing School With Innovation of Guangdong Ocean University: GDOU2016050249.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.btre.2018.e00262.

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