Molecular cloning and characteristics analysis of Pmtgfbr1 from Pinctada fucata martensii

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\section*{A B S T R A C T}

\textit{Pinctada fucata martensii} is cultured for pearl production. Growth improvement has received considerable research interest. Transforming growth factor $\beta$ type I receptor (Tgfbr-I), which is involved in signals transmission of transforming growth factor beta (TGF-$\beta$), participates in cell proliferation and growth. In this study, we characterized a Tgfbr-I gene which encoded Tgfbr-I from \textit{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 Tgfbr-I structure (extracellular receptor ligand domain, transmembrane domain, and cytoplasmic tyrosine kinase catalytic domain). Pmtgfbr1 is expressed in all 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.

\section*{1. Introduction}

The transforming growth factor $\beta$ (TGF-$\beta$) superfamily comprises bone morphogenetic proteins, activins, TGF-$\beta$s, and other related factors \cite{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 \cite{3–5}. TGF-$\beta$ family members transmit signals through signaling systems that involve types I and II serine/threonine kinase receptors \cite{6–8}. In receptor activation, the TGF-$\beta$ type I receptor (Tgfbr-I) mainly acts downstream of the TGF-$\beta$ type I receptor (Tgfbr-II) and sometimes determines the specificity of intracellular signals \cite{9,10}.

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

\textit{Pinctada fucata martensii} (synonymous to \textit{P. fucata} and \textit{P. martensii}) as an important species for pearl culture is widely studied for its biomineralization and immune system for the pearl production purpose \cite{24,25}. The TGF-$\beta$ signal and receptor genes of the pearl oyster \textit{P. fucata martensii} have been also analyzed \cite{26,27}. Most studies showed that TGF-$\beta$ signal pathway is associated with biomineralization in \textit{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 Tgfbr-I gene of \textit{P. fucata martensii} and estimated the relationship between Pmtgfbr1 and growth traits of pearl oysters.

\section*{2. Methods and materials}

\subsection*{2.1. Animals and sample collection}

Pearl oysters \textit{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),
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/orffinder). 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 Pmtgfbr1. Table 1 presents the specific primers used in this analysis. Transcripts were relatively quantified through 2-ΔΔCT method with GAPDH as the internal control [28,29].

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-ΔΔCT 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 anatina (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. anatina (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 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_recip 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_recip, GS: GS motif, S_TKc: serine/threonine protein kinases.
Multiple-sequence alignment of Pmtgfr1 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 martensi (AA98699.1), Crassostrea gigas (ERK41469.1), 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 Pmtgfr1 (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 Pmtgfr1 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 martensi tgfbr1 (AA98699.1), 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 1 receptor (AA83216.1), Danio rerio transforming growth factor-beta receptor type Ib (ABR20510.1), H. sapiens bone morphogenetic protein receptor, type IA (EAW80320.1), Danio rerio BMP receptor 1a protein (AAI63471.1), Homo sapiens BMPR1B (AAH147773.1), Danio rerio Bone morphogenetic protein receptor, type 1b (AAH16625.1), C. gigas activin-like type 1 receptor (CAC85263.1), H. sapiens ACVR1 protein (AAH33867.1), H. sapiens ALK-1 (CAAP0255.1), H. sapiens ACVR2B protein (AAH96245.1), D. rerio Acvr2b (AAI64219.1), C. gigas activin type II receptor (CAB92545.1), H. sapiens Bone morphogenetic protein receptor, type II (serine/threonine kinase) (AAH52985.1), C. gigas bone morphogenetic protein 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).
3.4. Construction of the Tgfr1 phylogenetic tree

To investigate the relationship between Pmtgfbr1 and other Tgfr-I and Tgfr-II receptors, a phylogenetic tree was constructed with 21 associated sequences from various phyla using the NJ method and 1000 bootstrap replications. Pmtgfbr1 showed a high degree of conservation with Tgfr-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, Gl, 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, Gl, 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 analysis and growth characterization experiments were utilized to validate the relationship of Pmtgfbr1 with growth of *P. fucata martensi*. 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).

![Fig. 5. Pmtgfbr1 expression levels in different P. fucata martensi tissues. ME, marginal zone of mantle; MC, central zone of mantle; A, adductor muscle; Gl, 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.](image)

![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).](image)

### Table 2

Correlation analysis between Pmtgfbr1 expression and growth traits of *P. fucata martensi*.

|                | Shell length | Shell height | Shell width | Total Weight | Tissue weight | Shell weight |
|----------------|--------------|--------------|-------------|--------------|---------------|--------------|
| Pmtgfbr1 expression levels | R  = 0.767** | R  = 0.794** | R  = 0.790** | R  = 0.767** | R  = 0.694** | R  = 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.

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 metazoa, 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 Tgfr-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 Tgfr1 proteins. The conserved serine/threonine kinase structures are crucial for determining the specificity of Tgfr-I for smad proteins [41]. The multiple-sequence alignment of the whole aa sequences of Pmtgfbr1 and other homologous Tgfr1 proteins revealed that the highly conserved serine/threonine protein kinases, identical SCGSGC sequences, and L45 loop are important features for signal transmission in the TGF-β pathway and that Tgfr-II phosphorylates Tgfr-I in the Ser of the SCGSGC motif [42–44]. Therefore, Pmtgfbr1 may have similar functions as Tgfr-I.

Phylogenetic analysis showed that Pmtgfbr1 has a high degree of conservation with Tgfr-I. Moreover, comparing the three-dimensional structure of the Pmtgfbr1 kinase domain with the kinase domain of Tgfr1 from *C. farreri* also showed that Pmtgfbr1 is highly conserved [23], providing additional evidence for the
potential function of this gene in Tgfr1 activation and the interactions between Tgfr1 and smad [45].

Pmtgfr1 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 Pmtgfr1 among all tissues, indicating that Pmtgfr1 has potential roles in muscle growth and regulation [13]. The TGF-β signaling pathway, which transmits signals via Tgfr1, is also expressed in the skeletal muscle of mammals, such as mice and humans, and is involved in myogenesis and muscle growth [47–50]. Tgfr1 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 Pmtgfr1 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, Pmtgfr1 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 Pmtgfr1 expression in adductor muscle and P. fucata martensii growth traits. Pmtgfr1 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) [24] also presented that Pmtgfr1 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 Pmtgfr1 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 Tgfr1 expression [23]. TGF-β may be involved in inhibiting skeletal muscle differentiation and in muscle growth [49].

5. Conclusion

Pmtgfr1 possesses the conserved domain of Tgfr1 and is expressed in all sampled tissues. There existed negative correlation between Pmtgfr1 expression levels and growth traits of P. fucata martensii. These results indicated that Pmtgfr1 genes negatively affect growth of P. fucata martensii.

Conflicts of interest

The authors declare no conflict of interest.

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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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