Acta Oceanol. Sin., 2018, Vol. 37, No. 2, P. 76–84
DOI: 10.1007/s13131-018-1164-x
http://www.hyxb.org.cn
E-mail: hyxbe@263.net

Cloning, expression profiling and promoter functional analysis of bone morphogenetic protein 2 in the tongue sole (*Cynoglossus semilaevis*)

MA Qian[1, 2], FAN Yanjun[1], ZHUANG Zhimeng[1, 3*], LIU Shufang[1, 2]

[1] Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao 266071, China

[2] Function Laboratory for Marine Fisheries Science and Food Production Processes, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266200, China

[3] Function Laboratory for Marine Biology and Biotechnolgy, Qingdao National Laboratory for Marine Science and Technology, Qingdao 266200, China

Received 3 March 2017; accepted 5 June 2017

Abstract

BMP2 plays crucial roles in vertebrate developmental process and acts as a bone inducer during osteogenesis. We present here the molecular cloning of *bmp2* cDNA from the marine flatfish *Cynoglossus semilaevis*, and the analysis of *bmp2* expression profiling and promoter function. The full length of *bmp2* cDNA sequence is 2 048 bp, which encodes a protein of 422 amino acids. Tissue expression distribution of *bmp2* was examined in 14 tissues of mature individuals by quantitative real time PCR (qRT-PCR). The results revealed that *bmp2* was expressed ubiquitously, and the highest expression level was detected in the spinal cord. Moreover, *bmp2* expression levels were detected at 15 sampling time points of early developmental stages (egg, larva, juvenile and fingerling stages). The highest expression level of *bmp2* was observed at the gastrula stage, which was about ten times higher than those at the other three embryo stages. Whole-mount *in situ* hybridization showed that the *bmp2* signal was strongly detected at the location of the crown-like larval fin, heart and liver, and slightly expressed in the notochord at one day post hatch (dph); then the expression of *bmp2* started to be concentrated in notochord at three dph. Subsequently, we characterized the 5′-flanking region of *bmp2* by testing the promoter activity by Luciferase reporter assays. Positive regulatory region was detected at the location of –179 to +109. The predicted transcription factor binding sites (E-box binding factors, zinc finger transcription factor, etc.) in this region might participate in the transcriptional regulation of the *bmp2* gene.

Key words: cloning, gene expression pattern, promoter transcriptional activity, bone morphogenetic protein, *Cynoglossus semilaevis*, early developmental stages

Citation: Ma Qian, Fan Yanjun, Zhuang Zhimeng, Liu Shufang. 2018. Cloning, expression profiling and promoter functional analysis of bone morphogenetic protein 2 in the tongue sole (*Cynoglossus semilaevis*). Acta Oceanologica Sinica, 37(2): 76–84, doi: 10.1007/s13131-018-1164-x

1 Introduction

Bone morphogenetic proteins (BMPs), belonging to the transforming growth factor beta (TGF-β) superfamily, were initially discovered by their ability to induce endochondral osteogenesis *in vivo* (Urist et al., 1979). To date, more than 30 members of the BMP family have been identified; numerous studies have demonstrated the efficacies of these BMP members in inducing bone formation (Kang et al., 2004; Cheng et al., 2003; Ducy and Karsenty, 2000). BMP2 has been extensively studied due to its predominant position within the BMP family and essential role in chondrogenesis and osteogenesis. BMP2 is required for mammalian osteogenesis, inducing osteoblast differentiation (Huang et al., 2002). For instance, Burkus et al. (2003) reported the positive effect of BMP2 on human long bone fracture repair, which includes formation of both cartilage and bone. Endogenous BMP2 was shown to play a pivotal role in formation of the mouse long bone (Kugimiya et al., 2005). The effect of BMP2 in chondrocyte and osteoblast differentiation and maturation has also been demonstrated in lower vertebrates. Quint et al. (2002) showed the involvement of BMP2 in lepidotrichia differentiation. It has been reported by Smith et al. (2006) that misexpression of *bmp2b* could induce ectopic bone formation within the regenerate of zebrafish caudal fin.

In addition to bone formation, BMPs have been demonstrated to be pleiotropic factors involving various growth and differentiation processes (Balemans and Van Hul, 2002; Hogan, 1996). The fundamental function of BMP2 in vertebrate embryonic development is directly involved in processes of dorsal–ventral axis specification (Graff, 1997). In zebrafish, *bmp2* participated in the induction and maintenance of ventrolateral cell fate during early development (Kishimoto et al., 1997). Furthermore, Hammond et al. (2009) also demonstrated a critical late role for *bmp2b* in the morphogenesis of semicircular canals in the zebrafish inner ear.

The important role of BMP2 in growth and development processes have contributed to the growing interest in understanding

Foundation item: The Special Scientific Research Funds for Central Non-profit Institutes, Chinese Academy of Fishery Sciences under contract No. 2016RC-LX02; the National Natural Science Foundation of China under contract No. 31201981.
*Corresponding author, E-mail: zhuangzm@ysfri.ac.cn

regulatory mechanism of cartilage and bone development in marine fish. So far the *bmp2* gene has been cloned from various teleost species, however, studies regarding the role of *bmp2* in development have been rarely reported except for the very few organisms such as zebrafish, senegalese sole and gilthead sea bream (Fernández et al., 2011; Marques et al., 2014; Marques et al., 2016). In terms of the activities of BMP2 during growth and development in fish, the aim of the work was to provide new insights into the evolutionary relationship between *bmp2* and other *bmp* members, and to investigate *bmp2* gene expression profiles and protein functions.

In this study, our goal is to gain insight into the genetic characteristics and functions of BMP2 in the tongue sole (*Cynoglossus semilaevis*). As the first step, the full length *bmp2* cDNA was cloned by homology cloning and the rapid amplification of cDNA ends polymerase chain reaction (RACE-PCR). Spatial- and temporal-expression analysis of *bmp2* during early developmental stages was performed by using qRT-PCR and whole-mount *in situ* hybridization. In order to explore transcriptional regulatory mechanism of the elaborated control of *bmp* gene expression, promoter activity of 5′-flanking region of *bmp2* gene was tested by Luciferase reporter assays. Relative results will provide new information to clarify the role of *bmp* transcription regulation in accomplishing functions related to skeletal development. Our findings would help understanding the role of *bmp2* in regulating early development of this flatfish at the molecular level.

2 Materials and methods

2.1 *Experiment design and sample source*

A total of 12 three-year old adult fish (six females and six males) were used to detect the *bmp2* tissue expression distribution. After being rapidly dissected from these live individuals, 14 tissues (brain, cartilage, dorsal fin, gill, gonad, heart, intestine, kidney, liver, muscle, skin (with scales), spleen, spinal cord and stomach) were immediately frozen in liquid nitrogen and kept at –80°C until use.

In order to examine the ontogenetic expression pattern of *bmp2* at early developmental stages, embryos and immature fish (larvae, juveniles and fingerlings) were used to supply samples for quantitative real time PCR (qRT-PCR) and whole-mount *in situ* hybridization analysis. The cultivation temperature for fertilized eggs was kept at 22°C. Fish samples were collected under the condition of empty stomach in the early morning. Samples for qRT-PCR are shown in Table 1, i.e., 20 eggs were collected at each egg stage (Multi-cell, Gastrula, Embryonic and Pre-hatching stage) and developmental stages were identified according to Wan et al. (2004), six individuals at each early developmental stage (larvae, juveniles and fingerlings) were also randomly collected. The whole body of these samples was frozen in liquid nitrogen for RNA extraction. Samples for whole-mount *in situ* hybridization (newly hatched, one and three dph) were collected and fixed in 4% paraformaldehyde (PFA) for 12–16 h, dehydrated with solutions with different ratios of ethanol (30%, 50% and 70%), and stored in 70% ethanol at 4°C.

2.2 *The first-strand cDNA synthesis*

Total RNA was extracted from frozen tissues of adult fish as well as samples from different early developmental stages using TRIzol Reagent (Invitrogen, USA) according to the manufacturer's instructions. The isolated RNA samples were suspended in DEPC-treated water, quantified using NanoVue^{TM} (GE Healthcare) at A_{260} nm and A_{280} nm, and then analyzed for integrity on agarose gel. The first-strand cDNA was synthesized from total RNA using PrimeScript^{TM} RT reagent Kit with gDNA Eraser (Takara Bio., China) following the manufacturer's instructions. The cDNA synthesis included a genomic DNA elimination reaction and the RT Primer Mix contained both Oligo dT Primer and Random 6 mers.

2.3 *Cloning of bmp2 cDNA*

According to the conserved sequences of *bmp2* gene from other teleost species, a pair of degenerate primers, BMP2-F/R (Table 2), was designed to enable cloning of the corresponding partial fragments of *bmp2* cDNA. PCR amplification was performed in a typical reaction; the condition was one initial denaturing step of 3 min at 94°C, followed by 32 cycles of 30 s at 94°C, 30 s at 53–58°C, 30 s at 72°C, and a final 10 min at 72°C.

Table 1. *Cynoglossus semilaevis* at different early developmental stages

Developmental stages	Embryo (hpf)			Larva (dph)			Juvenile (dph)	Fingerling (dph)
	Multi-cell stage	Gastrula stage	Embryonic stage	Pre-hatching stage	Early-stage larvae	Late-stage larvae		
Sampling time	3	13	22	32	1, 2	3, 4, 5, 10	20, 30, 40, 50	90
Sampling number	20 eggs at each developmental stage			six individuals at each developmental stage				

Note: hpf is hours post fertilization and dph days post hatching.

Based on the obtained partial fragments of *bmp2* cDNA, four specific primers, BMP2-5′-OUTER/INNER and BMP2-3′-OUTER/INNER (Table 2) were designed for amplification of the cDNA ends of the *bmp2* gene by using the 5′-Full RACE Kit and 3′-Full RACE Core Set Ver.2.0 (Takara Bio., China) following the manufacturer's instructions.

All the amplified fragments of the expected sizes were purified with a E.Z.N.A. Gel Extraction Kit (OMEGA, USA), cloned into a pMD18-T vector (Takara Bio., China), then transformed into *Escherichia coli* DH5α and sequenced by the Beijing Genomics Institute (BGI, Shenzhen, China).

2.4 *Sequence and phylogenetic analysis*

The full length cDNA of *bmp2* gene was assembled by aligning the overlapping fragments and the primer sequences. The signal peptides were predicted with Signalp 4.1 (http://genome.cbs.dtu.dk/services/SignalP). Putative domains and possible N-glycosylation sites were identified by PROSITE (http://www.expasy.org/prosite) (de Castro et al., 2006). Promoter prediction of *bmp2* gene was performed by BDGP (http://www.fruitfly.org/seq_tools/promoter.html), and putative transcription factor binding sites (TFBS) were identified by Version 8.3 of TRANSFAC (Messeguer et al., 2002) and the MatInspector program (http://www.genomatix.de).

Putative *bmp2* amino acid sequences of *C. semilaevis* and other vertebrates were used to construct a phylogenetic tree using the Neighbor-Joining (NJ) method with MEGA Version 5.1. The ClustalW program was employed to align all the sequences with the default option.

Table 2. Oligonucleotide primers used in this study

Name	Primer sequences (5′ to 3′)	Amplification target
BMP2-F	GGTGCCGCAGTACATGGTGGAC	cDNA fragment of *bmp2*
BMP2-R	GCAGCCACATCCCTCCACAAC	
BMP2-5′-OUTER	GCTCGCCTTGTGCTGTTGCTTCC	5′ RACE of *bmp2*
BMP2-5′-INNER	GTTGGGGTGTGCTTCTCCAGTGA	
BMP2-3′-OUTER	TACCACGCCTTTTATTGCCAC	3′ RACE of *bmp2*
BMP2-3′-INNER	CTGGATGAAAATGGGAAGGTC	
BMP2-PRO-F1	GATTAAGTTGGCTTGCCCTTCTC	5′-flanking region of *bmp2*
BMP2-PRO-F2	GAATTAGACGTGCGTAAATGTGCA	
BMP2-PRO-F3	ACACTTAGATCGGTCCTACCTTCAC	
BMP2-PRO-F4	GGTATATGAGAACGGGATTTATAATAGTG	
BMP2-PRO-F5	TATACATGACCGTCAAAGCCTCTG	
BMP2-PRO-F6	TTTGTTCCACTTCAAAGTGTTCTTTC	
BMP2-PRO-R	CCGATTCGCTGTATTTCCTCC	
BMP2-RT-F	CAGGACTCTCTAAGCCGCTG	expression of *bmp2*
BMP2-RT-R	CCGAACATGCCTACTACGCT	
BMP2-HY-F	GCTTCGCCTTCTTAATATGTTTGGA	whole-mount *in situ* hybridization of *bmp2*
BMP2-HY-R	ATGTCGCTCCTCCACCTCT	
EF1A-F	GACAAACTGAAGGCHGAGCG	expression of *ef1a*
EF1A-R	CAGCCTGAGAGGTTCCAGTGAT	
18S-F	CCTGAGAAACGGCTACCACATCC	expression of *18s*
18S-R	CCAATTACAGGGCCTCGAAAG	

2.5 *Promoter activity of the 5′-flanking region of bmp2 gene*

2.5.1 *Construction of luciferase-reporter gene vector for bmp2 gene promoter*

A DNA fragment with the length of 1 863 bp (–1754 to +109) was cloned from the 5′-flanking regions of *bmp2* gene (Gene ID: 103381057). Promoter deletion experiment was designed to monitor the promoter activity of the 5′-flanking regions. DNA fragments with a series of nested deletions (gradually truncated 5′-end with the predicted promoter sequence) were generated by using the primers listed in Table 2, which were designed according to *C. semilaevis bmp2* sequence as well as the promoter prediction results from TRANSFAC and MatInspector programs. A total of six plasmids were constructed by inserting DNA fragments of various sizes from the 5′-flanking region of *bmp2* gene into the region between the *Hind*III and *Xho*I sites of the pGL4.10 vector (Promega, E665A). The sequences and orientations of the constructs were verified by nucleotide sequencing.

2.5.2 *Cell culture and transient transfection*

ZF4 cells (purchased from ATCC) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS, Lifetechnologies), 100 U/mL penicillin (Lifetechnologies) and 100 U/mL streptomycin (Lifetechnologies). Cells were seeded to 96-well plates for 24 h before transfection. When the cell grew to 90%, equivalent amounts of plasmids were transfected into ZF4 cells following the instructions of transfection reagent (Lipofectamine® 2000, Thermo Fisher Scientific). pGL4.51 (Promega, E132A) was used as a reference (positive control), while pGL4.10 (Promega, E665A) without promoter was used as negative control. The ratio of target plasmids to pGL4.51 or pGL4.10 were both 1:1.

2.5.3 *Detection of luciferase activity*

After transfection for 48 h, luciferase activity was detected by using Luciferase Reporter Gene Assay Kit (Beyotime) in accordance with the manufacturer's instructions. Three replications were set up for each sample from *bmp2* gene, and relative light unit (RLU) was detected by EnVision® Multilabel Reader (PerkinElmer, USA).

2.6 *Quantitative real time PCR*

The qRT-PCR was conducted to determine *bmp2* tissue expression distribution in adult fish as well as their dynamic expression patterns at early developmental stages. The primers BMP2-RT-F/R, EF1A-F/R and +-F/R were used for amplifying the *bmp2*, elongation factor 1-alpha (*ef1a*) and 18S rRNA (*18s*) fragments, respectively (Table 2). The qRT-PCR was conducted on a 7500 ABI Real time PCR system (Applied Biosystems, USA). Amplifications were performed in a 20 μL final volume containing 1 μL cDNA sample, 10 μL SYBR® *Premix Ex Taq*™ (Perfect Real Time) (Takara Bio., China), 0.4 μL ROXII, 0.4 μL of each primer and 7.8 μL ddH_2O. A negative control was always included. PCR amplifications were performed in triplicate using the following conditions: initial denaturing at 95°C for 10 s, followed by 40 cycles of 5 s at 95°C and 34 s at 60°C. A dissociation protocol was always performed after thermocycling to determine target specificity. Expression of *ef1a* was used as the internal control for *bmp2* tissue expression distribution analysis, and *18s* was used as the internal control for the *bmp2* ontogenetic expression patterns (Ma et al., 2015). The ratio changes in the target genes relative to the control genes (*ef1a* and *18s*) were determined by the $2^{-\triangle\triangle CT}$ method (Livak and Schmittgen, 2001) and the transcript level was described in terms of its relative concentration ($RC_{target}/RC_{control}$).

2.7 *Whole-mount in situ hybridization*

For *in situ* hybridization, plasmids (pGEM-T vector, Promega) containing the fragment of *bmp2* cDNA (619 bp) was linearized by restriction endonuclease digestion using *Nco*I (NEB Biolabs). Sense and antisense digoxigenin (DIG)-labeled RNA probes were synthesized from the plasmids with SP6 or T7 transcriptase by using DIG RNA Labeling Kit (Roche) following the manufacturer's instructions. Whole-mount *in situ* hybridization

procedures followed the standard protocols according to Holland et al. (1996). Samples at different developmental stages were first rehydrated, washed, treated with Proteinase K, followed by 4% PFA refix. Specimens were incubated for 3 h at 60°C in prehybridization buffer and then hybridized with the labelled probes for 16 h at 60°C. After hybridization, the larvae were washed and blocked with blocking reagent (Roche) at room temperature for 2 h, then incubated with an alkalinephosphatase-conjugated anti-DIG antibody (1:2 000 dilution) over night at 4°C. Finally, excess anti-DIG antibodies were removed by rinsing, and signals were visualized by incubation in NBT/BCIP reagents (Roche) in dark. Specimens were observed and photographed using a Nikon microscope (SMZ1500).

2.8 *Statistical analysis*

All data were expressed as mean±SD and analyzed by one-way ANOVA (analysis of variance) to determine significant differences between means using SPSS 16.0. Values were considered statistically significant when $P<0.05$.

3 Results

3.1 *Characteristics and phylogenetic analysis of bmp2 gene*

As shown in Fig. 1, *C. semilaevis bmp2* cDNA is 2 048 bp and contains a 5′-untranslated region (UTR) of 442 bp, an open reading frame (ORF) of 1 269 bp and a 3′ UTR of 337 bp (GenBank ID: KC422338). The predicted amino acid sequence of the *bmp2* cDNA consists of 422 amino acid residues with a 23 amino-acid signal peptide. The predicted molecular weight of BMP2 is 47.47 kDa, and the theoretical isoelectric point (pI) is 8.65. Seven cysteine residues and an RVSR sequence were found in the *bmp2* amino acid sequence.

A phylogenetic tree was constructed by the Neighbor-Joining method to investigate relationships of *bmp2* genes in the listed species, based on the genetic distances calculated with the Poisson correction model (Fig. 2). Results showed that lineages were composed of two branches: teleost branch and mammal branch. Within the lineage of teleost *bmp2* branch, *C. semilaevis* formed a single branch out of the branch composed of 13 teleost species (five from Cichlidae, two from Tetraodontidae, and one each from Pomacentridae, Sciaenidae, Nothobranchiidae, Sparidae, Carangidae and Paralichthyidae). Basically, the phylogenetic relationship result among vertebrates was consistent with the traditional taxonomy.

3.2 *Functional analysis of the 5′-flanking region of bmp2 gene*

The DNA fragment with the total length of 1 873 bp was obtained from 5′-flanking region of the *bmp2* gene. To test the promoter activity of the 5′-flanking region, gradual deletions were performed according to promoter prediction results. As a result, a total of six promoter fragments were obtained and inserted into the pGL4.10 vector, sequencing results showed that all these sequences were correctly inserted into the vector. Luciferase reporting assay showed that activities of *bmp2* promoter with the total length of 1 873 bp could be successfully detected. No significant variation in promoter activity was detected by the gradual deletion of 5′ ends of the promoter fragments of *bmp2* gene (Fig. 3), which minimize the positive regulatory region of *bmp2* to –179 to +109.

A TATA box was found –1 505 bp upstream from the start codon of the *bmp2* gene by TRANSFAC. MatInspector was used for searching for transcription factor binding sites in the positive regulatory region of *bmp2* gene (–179 to +109). The results indicated that several factors, such as E-box binding factors, zinc finger transcription factor and binding site for a Pbx1/Meis1 heterodimer were located in the 5′-flanking region of *C. semilaevis bmp2* (Fig. 4).

3.3 *Gene expression pattern of C. semilaevis bmp2*

3.3.1 *Tissue expression distribution of bmp2*

Tissue expression distribution of *bmp2* in *C. semilaevis* adults was determined by qRT-PCR. As shown in Fig. 5, the highest *bmp2* expression level was detected in the spinal cord, similar high expression levels were found in the spleen, skin (with scales), intestine, gill, cartilage and brain; medium expression levels were detected in the liver, stomach, kidney, gonad and muscle; the lowest expression level was detected in the heart.

3.3.2 *Temporal-expression of bmp2 during early development*

As indicated in Fig. 6, *bmp2* was highly expressed at the gastrula stage during early embryo development, and the expression level was about ten times higher in comparison with those at other egg stages. An increasing trend of *bmp2* expression was detected from late embryo stages to early larva stages. The *bmp2* expression levels stayed constant after 30 dph.

3.3.3 *Spatial-expression of bmp2 at early larva stages*

Expression patterns of *C. semilaevis bmp2* at early larva stages were examined by whole-mount *in situ* hybridization. As shown in Fig. 7, the *bmp2* expression in newly hatched larvae was quite low. At one dph, *bmp2* started to be strongly expressed in the crown-like larval fin, heart and liver; slightly expressed in the notochord. The expression of *bmp2* started to be concentrated in notochord but much less expressed in other locations at three dph.

4 Discussion

4.1 *Characteristics of C. semilaevis bmp2 gene*

In general, members of the BMP family exhibit similar gene characteristics. For instance, the C-terminal (comprising seven cysteine residues) was found conserved among BMP members, similar cleavage sites RXXR (related to sequential cleavage of the synthesized nonactive precursor protein and release of mature ligand) could also be found at certain location of the BMP amino acid sequences (Bragdon et al., 2011; Heng et al., 2010). In this study, the cleavage site RVSR was observed in *C. semilaevis bmp2* amino acid sequence. Similarly, our previous study showed that cleavage sites (RTTR and RSIR) were found in *C. semilaevis bmp6* and *bmp7* genes, and their locations were conserved in the sequences (Ma et al., 2017).

The presence of two BMP2 isoforms (BMP2a and BMP2b) were observed in *Astyanax mexicanus*, *Danio rerio* and *Oryzias latipes*, while only one has been reported in the genome of other vertebrates including *C. semilaevis*. As shown in Fig. 2, the three *bmp2b* genes clustered with other *bmp2* genes to form a teleost *bmp2* lineage, and the *D. rerio bmp2a* formed a single branch out of the teleost *bmp2* cluster. These results supported the view that *bmp2b* would represent the *bmp2* orthologous gene (Marques et al., 2016). One possible reason for the presence of *bmp2a* and *bmp2b* isoforms might be the gene duplication event in fish (*D. rerio* and other closely related species) (Sato and Nishida, 2010; Talbot and Hopkins, 2000; Taylor et al., 2001).

Transcription regulation is one of the most important steps in the regulation of gene expression. However, limit is known about

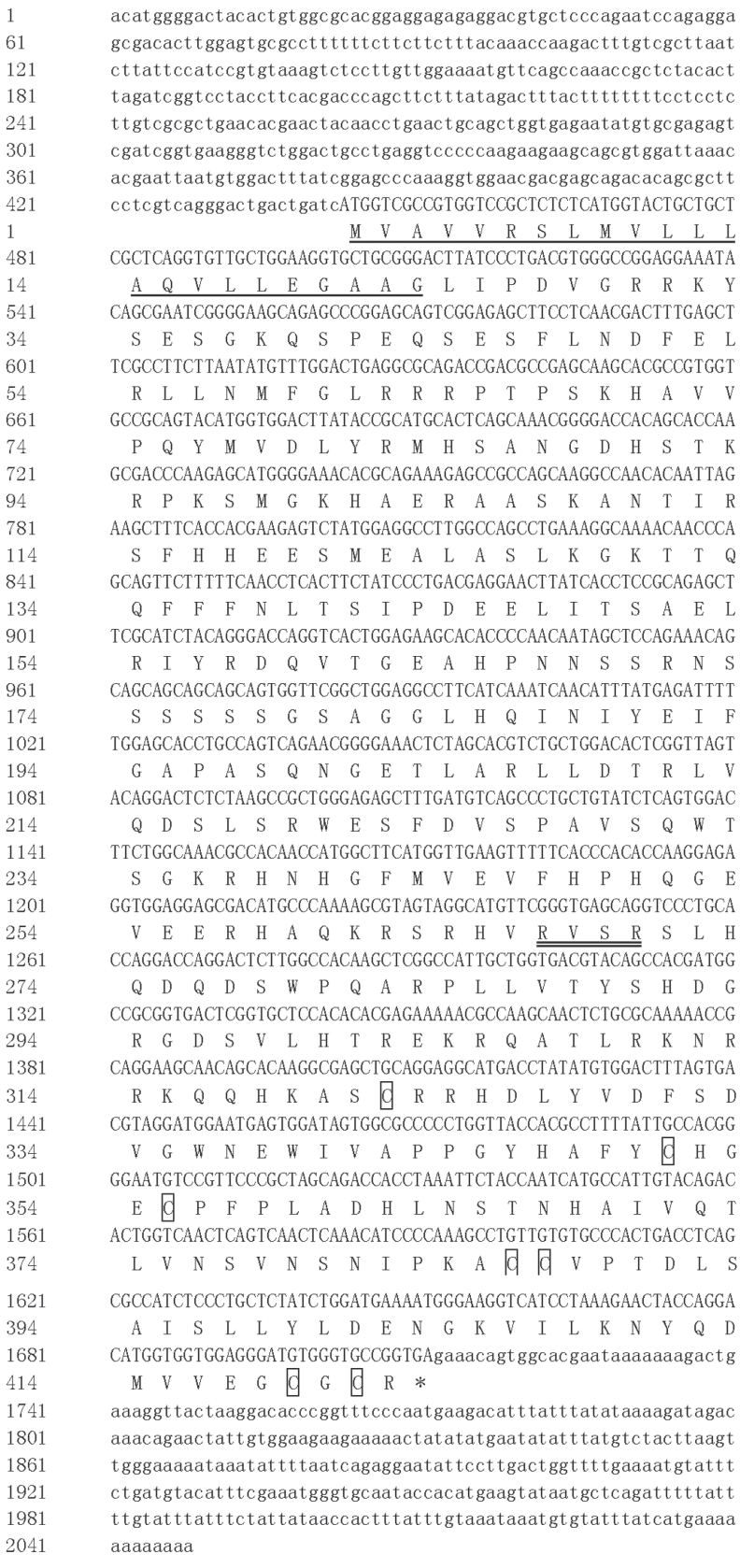

Fig. 1. Nucleotide and deduced amino acid sequence of *Cynoglossus semilaevis bmp2* cDNA. The deduced amino acid residues are represented as single letter abbreviations and numbered from the initiating methionine. The stop codon is marked by an asterisk. The signal peptide is underlined and the conserved cysteine residues are in bold boxes. The predicted protease-recognition sequence (RVSR) of the *bmp2* is double underlined. The sequence was submitted to GenBank under accession number KC422336.

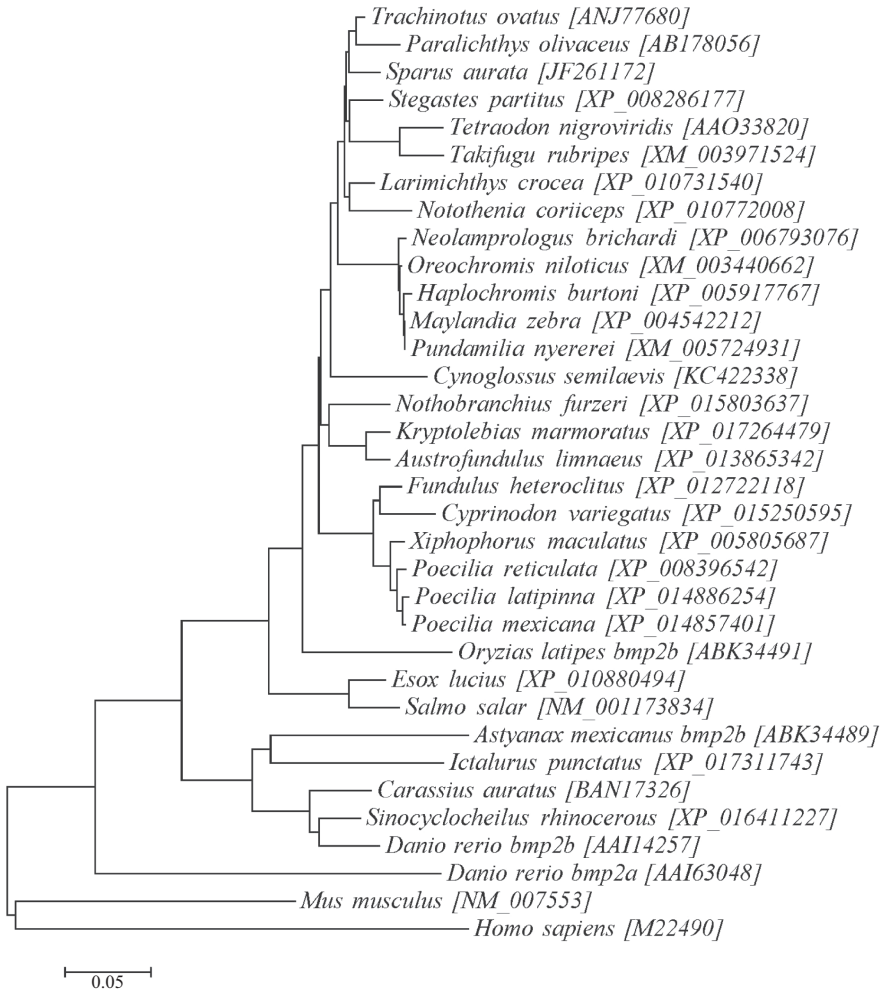

Fig. 2. Phylogenetic tree of *bmp2* amino acid sequences based on Neighbor-Joining (NJ) method. The bootstrap confidence values shown at the nodes of the tree are based on a 1 000 bootstrap procedure, and the branch length scale in terms of genetic distance is indicated below the tree.

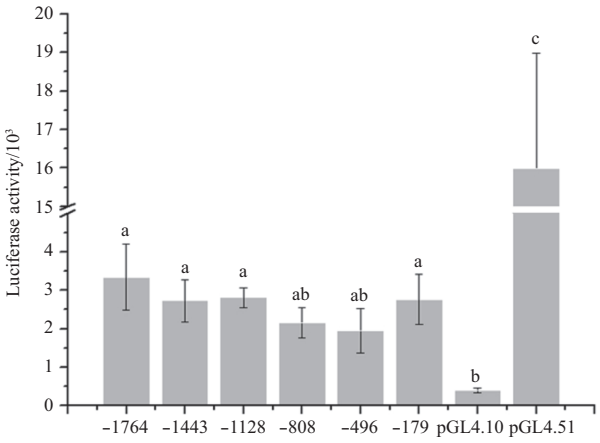

Fig. 3. Promoter activity of the *Cynoglossus semilaevis bmp2* gene 5′- flanking regions. –1764, pGL4.10 (–1764 to +109); –1443, pGL4.10 (–1443 to +109); –1128, pGL4.10 (–1128 to +109); –808, pGL4.10 (–808 to +109); –496, pGL4.10 (–496 to +109); –179, pGL4.10 (–179 to +109); pGL4.10, negative control; pGL4.51, positive control. All data are expressed as the mean±SD (n=3) and analyzed by one-way ANOVA. The letters indicate significant differences (P<0.05).

transcriptional regulation of BMP2 by bone- and cartilage-related transcription factors (TFs). In addition, predicted function determined from their primary structure is not sufficient to understand the mode of gene expression regulation at the transcriptional level. In this study, we analyzed the promoter activity of 5′-flanking region of *bmp2* gene, since promoter is the best-characterized transcriptional regulatory element. Positive regulatory region of *C. semilaevis bmp2* was detected at the location of –179 to +109. In comparison with our previous results of *C. semilaevis bmp6* and *bmp7*, these three *bmp* genes displayed different promoter features, i.e., the positive regulatory region of the *bmp6* gene located at –272 to +28, while *bmp7* contained one potential region fairly far upstream of the start codon (–740 to –396).

Regulatory elements and transcription factors involved in the control of *bmp2* expression were also identified in this study. A TATA box was found in the 5′-flanking region of the *bmp2* gene. However, the investigation of TATA box around the transcription initiation sites was not performed in this study. As reported by Dathe et al. (2009), the *bmp2* gene was shown to be flanked by long regions without nearby genes that may contain important regulatory elements. The long-range regulators controlling BMP2 transcription have also been reported by Chandler et al. (2007). A survey of *bmp2* genes in teleosts (such as zebrafish, gilthead seabream and spotted gar) has failed to identify TATA boxes up-

Fig. 4. Nucleotide sequences of positive regulatory region of *bmp2* gene. Potential transcription factor binding sites are boxed. Capitals in the boxes indicate the core sequences.

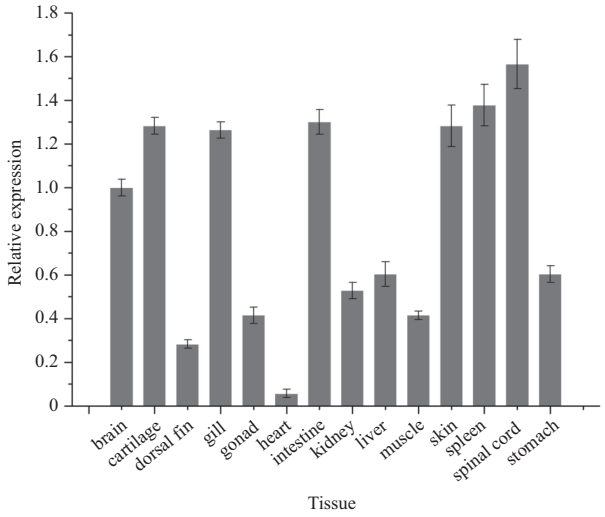

Fig. 5. Expression of *bmp2* mRNA in various *Cynoglossus semilaevis* tissues. The *bmp2* mRNA expression levels are expressed as a ratio relative to the *ef1a* mRNA levels in the same samples. A relative abundance of 1 was set arbitrarily for the mRNA level of *bmp2* in the brain. Values are expressed as mean±SD (n=3). Skin including scales is considered a mixed tissue.

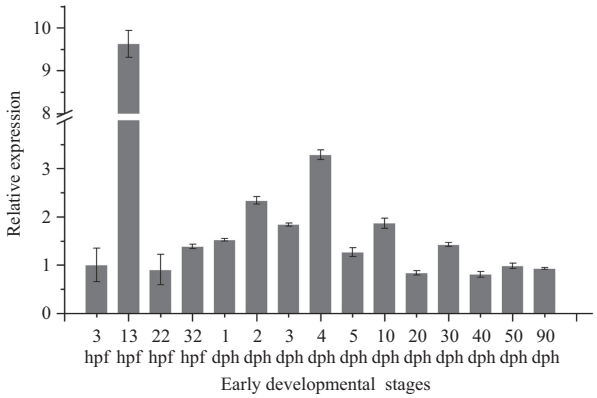

Fig. 6. The quantitative RT-PCR analysis of the *bmp2* mRNA expression levels at various early developmental stages of *Cynoglossus semilaevis*. Hpf represents hours post fertilization and dph days post hatching. *Bmp2* mRNA expression levels were expressed as a ratio relative to the *18s* levels in the same samples. A relative abundance of 1 was set arbitrarily for the mRNA level of *bmp2* at 3 hpf. All data are expressed as the mean±SD (n=3).

stream the TSS, suggesting that the presence of TATA-less promoters in BMP2 genes is a common feature (Marques et al., 2016). Lack of consensus TATA box around the transcription initiation sites in *bmp* genes has been demonstrated (Kawai and Sugiura, 2001; Simon et al., 2002), including human and mouse *bmp2* genes (Ghosh-Choudhury et al., 2001; Sugiura, 1999). It is now clear that only 10%–20% of mammalian promoters contain a functional TATA box, and TATA-less promoters can also regulate tissue-specific expression (Hochheimer and Tjian, 2003; Ling et al., 2009; Sandelin et al., 2007).

4.2 *Expression patterns of bmp2 gene*

The central role of *bmp2* subfamily in calcified tissues, particularly in scales, has been reviewed by Marques et al. (2016). High expression levels of *bmp2* gene in calcified tissues (i.e., bone, scales and caudal fin) were detected in *Solea senegalensis* and *Sparus aurata* (Marques et al., 2014; Rafael et al., 2006), which supported the well documented role of *bmp2* in tissue mineralization (Alexander et al., 2011; Wang et al., 1990). Similarly, *bmp2* exhibited the highest expression level in the mixed tissue (skin with scales) in this study, which contained a mixture of soft and calcified tissues. However, low level of *bmp2* in dorsal fin and high level in cartilage were detected in mature *C. semilaevis*, revealing an inconsistent pattern in tissue distribution.

High expression of *bmp2* in the brain, spinal cord and intestine of *C. semilaevis* and *D. rerio*, supported the important role of this gene in central (particularly in the enteric) nervous system formation (Chalazonitis and Kessler, 2012; Sato et al., 2010). Among the other tissues with high *bmp2* expression, gill was known to be important for processes such as osmoregulation and respiration, which the BMP signaling was reported to be involved with (Kültz, 2012). As reported by Rafael et al. (2006), high expression of BMP2 in the liver of *S. aurata* suggested a possible role for this protein in liver cell trans-differentiation. In this study, high expression of *bmp2* was not observed in the liver and heart of *C. semilaevis* adults, however whole-mount *in situ* hybridization assessed *bmp2* expression at these locations in two days old larvae. These results suggested that *bmp2* could be involved in early development of soft tissues such as heart and liver.

BMP2 has been implicated to be essential for appropriate embryonic dorsoventral patterning of zebrafish embryos during gastrulation (Xue et al., 2014). In this study, a significant increment of *bmp2* expression was detected in 13 hpf (hours post fertilization) embryos, corresponding to *C. semilaevis* gastrula stage. Similar results were also detected in *S. aurata*, revealing that the *bmp2* expression was strongly and transiently up-regulated at 10 hpf (gastrulation) (Rafael et al., 2006). Taking the previous demonstration that *bmp2b* represents the orthologous *bmp2* gene in zebrafish, significantly higher expression of *bmp2b* was

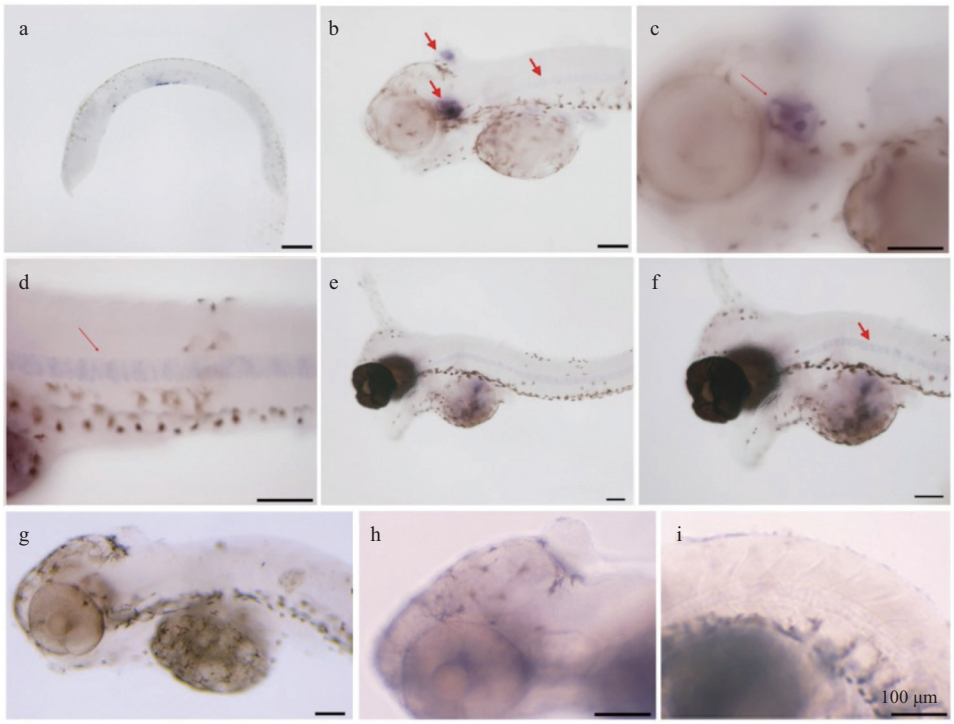

Fig. 7. Whole mount *in situ* hybridization of *bmp2* mRNA in *C. semilaevis* at early stages post hatching. Hybridization signals were reflected as bluish violet color. a. Newly hatched, b–d. 1 dph, e and f. 3 dph, and g–i. negative control, 1 dph.

also detected at pregastrula and gastrula stages when comparing to other embryo stages (Martínez-Barberá et al., 1997). In agreement with the observations in other higher vertebrates such as mouse and chicken, BMP2 was also detected at pregastrula and gastrula stages, when calcified structures were not yet formed (Schlange et al., 2000; Ying and Zhao, 2001). These findings suggested the important role of BMP2 in fish development at the onset of gastrulation, which is a decisive stage for cell fate and embryonic patterning (Rafael et al., 2006).

The *bmp2* expression presented in one-day old *C. semilaevis* was at levels similar to that observed in pre-hatching embryos (Fig. 6). Subsequently, highest *bmp2* mRNA levels were respectively detected at four and two days post hatching. Under this circumstance, preliminary investigation of *bmp2* location in *C. semilaevis* early larvae was performed by whole-mount *in situ* hybridization. The results revealed the potential role of *bmp2* in fin growth, as well as development of heart, liver and notochord.

5 Conclusions

The isolation of *bmp2* in this study, as well as the identification of transcription factors involves in the control of *bmp2* expression, provides encouragement for research of the *bmp* members in teleosts. According to expression pattern of the *bmp2* gene, we reported the potential role of *bmp2* in fin growth and development of notochord, which may help elucidating the role of *bmp2* in regulating bone formation and growth of *C. semilaevis*.

References

Alexander C, Zuniga E, Blitz I L, et al. 2011. Combinatorial roles for BMPs and Endothelin 1 in patterning the dorsal-ventral axis of the craniofacial skeleton. Development, 138(23): 5135–5146

Balemans W, Van Hul W. 2002. Extracellular regulation of BMP signaling in vertebrates: a cocktail of modulators. Developmental Biology, 250(2): 231–250

Bragdon B, Moseychuk O, Saldanha S, et al. 2011. Bone morphogenetic proteins: a critical review. Cellular Signalling, 23(4): 609–620

Burkus J K, Heim S E, Gornet M F, et al. 2003. Is INFUSE bone graft superior to autograft bone? An integrated analysis of clinical trials using the LT-CAGE lumbar tapered fusion device Journal of Spinal Disorders & Techniques, 16(2): 113–122

Chalazonitis A, Kessler J A. 2012. Pleiotropic effects of the bone morphogenetic proteins on development of the enteric nervous system. Developmental Neurobiology, 72(6): 843–856

Chandler R L, Chandler K J, McFarland K A, et al. 2007. *Bmp2* transcription in osteoblast progenitors is regulated by a distant 3′ enhancer located 156.3 kilobases from the promoter. Molecular and Cellular Biology, 27(8): 2934–2951

Cheng Hongwei, Jiang Wei, Phillips F M, et al. 2003. Osteogenic activity of the fourteen types of human bone morphogenetic proteins (BMPs). The Journal of Bone and Joint Surgery (American Volume), 85-A(8): 1544–1552

Dathe K, Kjaer K W, Brehm A, et al. 2009. Duplications involving a conserved regulatory element downstream of *BMP2* are associated with brachydactyly type A2. The American Journal of Human Genetics, 84(4): 483–492

de Castro E, Sigrist C J A, Gattiker A, et al. 2006. ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Research, 34(S2): W362–W365

Ducy P, Karsenty G. 2000. The family of bone morphogenetic proteins. Kidney International, 57(6): 2207–2214

Fernández I, Darias M, Andree K B, et al. 2011. Coordinated gene expression during gilthead sea bream skeletogenesis and its disruption by nutritional hypervitaminosis A. BMC Developmental Biology, 11: 7

Ghosh-Choudhury N, Choudhury G G, Harris M A, et al. 2001. Autoregulation of mouse BMP-2 gene transcription is directed by the proximal promoter element. Biochemical and Biophysical Research Communications, 286(1): 101–108

Graff J M. 1997. Embryonic patterning: to BMP or not to BMP, that is

the question. Cell, 89(2): 171–174
Hammond K L, Loynes H E, Mowbray C, et al. 2009. A late role for *bmp2b* in the morphogenesis of semicircular canal ducts in the zebrafish inner ear. PLoS One, 4(2): e4368
Heng S, Paule S, Hardman B, et al. 2010. Posttranslational activation of bone morphogenetic protein 2 is mediated by proprotein convertase 6 during decidualization for pregnancy establishment. Endocrinology, 151(8): 3909–3917
Hochheimer A, Tjian R. 2003. Diversified transcription initiation complexes expand promoter selectivity and tissue-specific gene expression. Genes & Development, 17(11): 1309–1320
Hogan B L. 1996. Bone morphogenetic proteins: multifunctional regulators of vertebrate development. Genes & Development, 10(13): 1580–1594
Holland L Z, Holland P W, Holland N D, et al. 1996. Revealing homologies between body parts of distantly related animals by in situ hybridization to developmental genes: amphioxus versus vertebrates. In: Ferraris J, Palumbi S R, eds. Molecular Zoology: Advances, Strategies, and Protocols. New York: Wiley, 473–483
Huang Weibiao, Rudkin G H, Carlsen B, et al. 2002. Overexpression of BMP-2 modulates morphology, growth, and gene expression in osteoblastic cells. Experimental Cell Research, 274(2): 226–234
Kang Q, Sun M H, Cheng H, et al. 2004. Characterization of the distinct orthotopic bone-forming activity of 14 BMPs using recombinant adenovirus-mediated gene delivery. Gene Therapy, 11(17): 1312–1320
Kawai S, Sugiura T. 2001. Characterization of human bone morphogenetic protein (BMP)-4 and -7 gene promoters: activation of BMP promoters by Gli, a sonic hedgehog mediator. Bone, 29(1): 54–61
Kishimoto Y, Lee K H, Zon L, et al. 1997. The molecular nature of zebrafish swirl: BMP2 function is essential during early dorsoventral patterning. Development, 124(22): 4457–4466
Kugimiya F, Kawaguchi H, Kamekura S, et al. 2005. Involvement of endogenous bone morphogenetic protein (BMP) 2 and BMP6 in bone formation. Journal of Biological Chemistry, 280(42): 35704–35712
Kültz D. 2012. The combinatorial nature of osmosensing in fishes. Physiology, 27(4): 259–275
Ling Fei, Wang Tao, Wei Liqiong, et al. 2009. Cloning and characterization of the 5′-flanking region of the pig *adiponectin* gene. Biochemical and Biophysical Research Communications, 381(2): 236–240
Livak K J, Schmittgen T D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods, 25(4): 402–408
Ma Qian, Feng Wenrong, Zhuang Zhimeng, et al. 2017. Cloning, expression profiling and promoter functional analysis of Bone morphogenetic protein 6 and 7 in tongue sole (*Cynoglossus semilaevis*). Fish Physiology and Biochemistry, 43(2): 435–454, doi: 10.1007/s10695-016-0298-z
Ma Qian, Zhuang Zhimeng, Feng Wenrong, et al. 2015. Evaluation of reference genes for quantitative real-time PCR analysis of gene expression during early development processes of the tongue sole (*Cynoglossus semilaevis*). Acta Oceanologica Sinica, 34(10): 90–97
Marques C L, Fernández I, Rosa J, et al. 2014. Spatiotemporal expression and retinoic acid regulation of bone morphogenetic proteins 2, 4 and 16 in Senegalese sole. Journal of Applied Ichthyology, 30(4): 713–720
Marques C L, Fernández I, Viegas M N, et al. 2016. Comparative analysis of zebrafish bone morphogenetic proteins 2, 4 and 16: molecular and evolutionary perspectives. Cellular and Molecular Life Sciences, 73(4): 841–857
Martínez-Barberá J P, Toresson H, Da Rocha S, et al. 1997. Cloning and expression of three members of the zebrafish Bmp family: *Bmp2a*, *Bmp2b* and *Bmp4*. Gene, 198(1-2): 53–59
Messeguer X, Escudero R, Farré D, et al. 2002. PROMO: detection of known transcription regulatory elements using species-tailored searches. Bioinformatics, 18(2): 333–334
Quint E, Smith A, Avaron F, et al. 2002. Bone patterning is altered in the regenerating zebrafish caudal fin after ectopic expression of sonic *hedgehog* and *bmp2b* or exposure to cyclopamine. Proceedings of the National Academy of Sciences of the United States of America, 99(13): 8713–8718
Rafael M S, Laizé V, Cancela M L. 2006. Identification of *Sparus aurata* bone morphogenetic protein 2: molecular cloning, gene expression and *in silico* analysis of protein conserved features in vertebrates. Bone, 39(6): 1373–1381
Sandelin A, Carninci P, Lenhard B, et al. 2007. Mammalian RNA polymerase II core promoters: insights from genome-wide studies. Nature Reviews Genetics, 8(6): 424–436
Sato T, Mikawa S, Sato K. 2010. BMP2 expression in the adult rat brain. The Journal of Comparative Neurology, 518(22): 4513–4530
Sato Y, Nishida M. 2010. Teleost fish with specific genome duplication as unique models of vertebrate evolution. Environmental Biology of Fishes, 88(2): 169–188
Schlange T, Andrée B, Arnold H H, et al. 2000. BMP2 is required for early heart development during a distinct time period. Mechanisms of Development, 91(1-2): 259–270
Simon M, Feliers D, Arar M, et al. 2002. Cloning of the 5′-flanking region of the murine bone morphogenetic protein-7 gene. Molecular and Cellular Biochemistry, 233(1-2): 31–37
Smith A, Avaron F, Guay D, et al. 2006. Inhibition of BMP signaling during zebrafish fin regeneration disrupts fin growth and scleroblast differentiation and function. Developmental Biology, 299(2): 438–454
Sugiura T. 1999. Cloning and functional characterization of the 5′-flanking region of the human bone morphogenetic protein-2 gene. Biochemical Journal, 338(2): 433–440
Talbot W S, Hopkins N. 2000. Zebrafish mutations and functional analysis of the vertebrate genome. Genes & Development, 14(7): 755–762
Taylor J S, Van de Peer Y, Braasch I, et al. 2001. Comparative genomics provides evidence for an ancient genome duplication event in fish. Philosophical Transactions of the Royal Society B: Biological Sciences, 356(1414): 1661–1679
Urist M R, Mikulski A, Lietze A. 1979. Solubilized and insolubilized bone morphogenetic protein. Proceedings of the National Academy of Science of the United States of America, 76(4): 1828–1832
Wan Ruijing, Jiang Yanwei, Zhuang Zhimeng. 2004. Morphological and developmental characters at the early stages of the tonguefish *Cynoglossus semilaevis*. Acta Zoologica Sinica (in Chinese), 50(1): 91–102
Wang E A, Rosen V, D'Alessandro J S, et al. 1990. Recombinant human bone morphogenetic protein induces bone formation. Proceedings of the National Academy of Science of the United States of America, 87(6): 2220–2224
Xue Yu, Zheng Xiudeng, Huang Lei, et al. 2014. Organizer-derived Bmp2 is required for the formation of a correct Bmp activity gradient during embryonic development. Nature Communications, 5: 3766
Ying Ying, Zhao Guangquan. 2001. Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse. Developmental Biology, 232(2): 484–492