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Research paper

Characterization and spatiotemporal expression analysis of nine bone morphogenetic protein family genes during intermuscular bone development in blunt snout bream

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ABSTRACT

Intermuscular bones (IBs) only exist in the myosepta of lower teleosts and its molecular mechanism remains to be clarified. Bone morphogenetic proteins (BMPs) have been demonstrated to be involved in various physiological processes, including bone and cartilage formation. In this study, we firstly obtained and characterized nine *bmp* genes for *Megalobrama amblycephala*, which belongs to Cyprinidae and have a certain amount of IBs. Sequence alignment and phylogenetic analysis both documented that the mature proteins of *M. amblycephala bmp* genes were highly conserved with other corresponding homologs, respectively, indicating that the function of each *bmp* gene has been conserved throughout evolution. As a step to characterize potential involvement of *bmp* genes in IB formation and development, spatiotemporal expressions of nine *bmp* genes (*bmp2a*, *bmp2b*, *bmp3*, *bmp4*, *bmp5*, *bmp7b*, *bmp8a*, *bmp14* and *bmp16*) were investigated during the key development stages of IBs. During the ossification process from stage I (the IBs haven't emerged) to stage IV (all of the IBs ossified in the tail with the mature morphology), the expression profiles revealed that *bmp16* was the most abundant transcript while *bmp4* had the lowest abundance. The mRNA levels of *bmp3*, *bmp4*, *bmp5* and *bmp8a* increased significantly at stage II, suggesting their roles in stimulating IB formation. The expression of *bmp7b* reached the highest level at stage III (the rapid period of IB development), suggesting potential involvement of *bmp7b* in promoting osteoblast differentiation. With the exception of *bmp7b* and *bmp16*, most *bmp* genes appeared a significant increase at IB maturation phase (stage IV), which means that they may play important roles in maintenance of IB morphogenesis. Spatial tissue distribution of *bmp* genes showed that most *bmp* genes were observed at the highest level in developing IBs at one year old fish. Spatiotemporal expression patterns suggest the potential key roles of these *bmp* genes in IBs formation and maintenance in fish, being as possible promoters or inhibitors.

1. Introduction

Bone morphogenetic proteins (BMPs), members of the transforming growth factor-beta (TGF-β) superfamily, are a group of multifunctional growth factors involved in various physiological processes, such as embryogenesis, tissue specification and organogenesis. To date, > 30 BMPs have been identified and more functions of them have been described (Ducy and Karsenty, 2000). The physiological importance of BMPs in bone metabolism and skeletal development has been well documented in mammals. For example, human BMP2 has been reported to induce structurally sound orthotopic bone in many studies, including femoral defects in rats, tibial and ulnar defects in rabbits, femoral defects in sheep, mandibular defects in dogs and spinal fusion in dogs (Riley et al., 1996). BMP3 has been regarded as a negative regulator of osteogenesis (Daluiski et al., 2001; Bahamonde and Lyons, 2001). BMP7 could promote and induce bone formation and bone repairing at ectopic fracture sites (Chen et al., 2001; Ngo et al., 2006). A large-scale association study in the pig revealed that BMP8 was very highly significantly associated with feet and leg structures (Fan et al., 2009). BMP14 (also known as GDF5) has been found to be implicated in promoting chondrogenic differentiation and inducing tendon (Tsumaki et al., 1999; Wolfman et al., 1997).

Abbreviations: IBs, intermuscular bones; BMPs, bone morphogenetic proteins; TGF-β, transforming growth factor-beta; ORFs, Open reading frames; PCR, Polymerase chain reaction; RNA, Ribonucleic acid; qRT-PCR, Quantitative real-time PCR; RT, Reverse transcriptase; NCBI, National centre for biotechnology information; UTR, untranslated region; GDF, Growth differentiation factor

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In recent years, research of BMPs in bone biology has been extended to fish models, especially the zebrafish (*Danio rerio*). Mowbray et al. (2001) described the mRNA expression patterns of *Bmp2b*, *Bmp4* and *Bmp7* in the developing zebrafish ear and provided evidence that BMPs are essential for the development of the vertebrate ear. Spatiotemporal expression patterns of *bmp2*, *bmp4* and *bmp16* in Senegalese sole (*Solea senegalensis*) and zebrafish revealed that all isoforms are expressed in calcified tissues, although to different extents (Marques et al., 2014; Marques et al., 2016). A transcriptional profile showed that *Bmp3* gene expression was down-regulated and *Bmp8a* was up-regulated during zebrafish caudal fin regeneration (Schebesta et al., 2006). In addition, *Bmp2a*, *Bmp2b*, *Bmp6* and *Bmp14* have also been documented to be involved in zebrafish fin development or regeneration (Crotwell et al., 2004; Quint et al., 2002; Ferlitsch et al., 2006; Crotwell et al., 2001). Zebrafish *swirl* mutant and *snailhouse* mutant, which are caused by mutations in the *Bmp2b* and *Bmp7a* gene respectively, are strongly dorsalized (Kishimoto et al., 1997; Dick et al., 2000), indicating that *Bmp2b* and *Bmp7a* are essential during early dorsoventral patterning. *Bmp16* was first identified in zebrafish based on high sequence similarity with *Bmp2/4*, and reported as a teleost-specific form initially (Feiner et al., 2009). However, subsequent research supported that *Bmp16* is not confined to teleost fish lineage but is largely absent from tetrapod genomes (Marques et al., 2016). The research on function of *bmp* genes in other fish species relating to bone biology is limited.

Intermuscular bones (IBs), which only exist in the myosepta in lower teleosts, are regarded as ossified myoseptal tendons developing directly from mesenchymal condensation (Patterson and Johnson, 1995). To date, many studies have described the morphology characters of IBs in fish species. The ossification and development of intermuscular bones have been reported in Japanese eel (*Anguilla japonica*) and several cyprinid fishes-*Cyprinus carpio*, *D. rerio*, *Hypophthalmichthys molitrix*, *Hemibarbus labeo* and *Megalobrama amblycephala* (Yao et al., 2014; Bing, 1962; Ke, 1965; Lü et al., 2012; Wan et al., 2014). In all these fishes, axial skeleton and appendicular skeleton have completely ossified before the emergence of intermuscular bones, consistent with previous view that the formation and development of IBs differs from that of other skeletons. IBs develop and ossify from the posterior part to anterior part in Cyprinidar species. However, in *A. japonica*, IBs appear first in the anterior part of the body then extend to posterior part (Yao et al., 2014). To date, studies on the molecular mechanism of IBs are limited. Wan et al. (2015, 2016) developed a larger miRNA and mRNA database for development of IBs in *M. amblycephala* by using the high throughout sequencing technology. However, little information is available relating to the regulation role of specific genes on IB development. The identified functions of *bmp* genes in bone metabolism and skeletal development of mammals and zebrafish suggest their possible roles in the development of fish IBs.

The blunt snout bream (*M. amblycephala*), belonging to the genus *Megalobrama* (Cypriniformes: Cyprinidae), has been widely farmed and consumed in China since the 1960s for its delicacy and strong adaptability (Ke, 1965). However, to some extent, the existence of IBs influences the farming and processing of *M. amblycephala*. In the present study, the cDNA sequences of nine *bmp* genes were obtained for *M. amblycephala*. To gain insight into the role of BMPs in IB development, a spatiotemporal expression of *bmp2a*, *bmp2b*, *bmp3*, *bmp4*, *bmp5*, *bmp7b*, *bmp8a*, *bmp14* and *bmp16* was described during different developmental stages of IBs. This is the first time discussing the effects for BMPs on the fish special bone-IB.

2. Materials and methods

2.1. Animals and tissue collection

All experiments were conducted in accordance with the guidelines of National Institute of Health Guide for the Care and Use of Laboratory Animals and approved by the Research Ethics Committee, Huazhong Agricultural University, Wuhan, China. The blunt snout bream *M. amblycephala* were obtained from the Fish Breeding Base of College of Fisheries (Hubei BaiRong Improved Aquatic Seed CO., LTD, Huanggang, 438800), Huazhong Agricultural University, Hubei province of China. After the specimens were anesthetized by MS-222 and sterilized with 75% alcohol, eight tissues (rib, IB, fin, brain, liver, muscle, heart and spleen) were immediately collected from the one-year-old (juvenile) and two-year-old (adult) *M. amblycephala*. In addition, samples (muscle tissue containing IBs in the tail) were collected from four key stages for IB development, which were identified by Wan et al. (2014, 2016). Their studies had identified four key stages of IB development for the *M. amblycephala* and had provided the working paradigm that was used in this study. All samples were rapidly separated, frozen in liquid nitrogen and stored at − 80 °C.

2.2. Molecular cloning of the M. amblycephala bmp cDNAs

A partial *M. amblycephala bmp3* cDNA sequence was identified from our previous transcriptomic data for *M. amblycephala* (GeneBank accession number: SRR233513). The full length *M. amblycephala bmp3* cDNA was obtained from total RNA from the fin by rapid amplification of cDNA end (RACE) method according to the manufacturer's instructions of the 5′ RACE System for Rapid Amplification of cDNA Ends, Version 2.0 Kit (Invitrogen, USA) and SMARTer™ RACE cDNA Amplification Kit (Clontech, Japan). Gene-specific primers for 5′- and 3′-RACE are presented in Table S1. Total RNA was extracted from the fin with using Trizol reagent (Invitrogen, USA) and reverse transcribed into the first-strand of cDNA using SuperScript II reverse transcriptase and the primer GSP-1. The synthesized cDNA was treated with RNase Mix to remove contaminant RNA and subsequently purified using DNA Purification System: GLASSMAX DNA isolation spin cartridges. Then the poly-C was added to the end of the purified cDNA using TdTase and dCTP. The cDNA with the dC tail was used as a template for first-round PCR with a forward primer GSP-2 and an abridged anchor primer (AAP; Invitrogen) as the reverse primer. In the second-round, as a nested PCR, a forward primer GSP-3 and an abridged universal amplification primer (AUAP; Invitrogen) were used to amplify the 5′ RACE fragment. The PCR products of the second-round were gel-purified, ligated into the T/A cloning vector pMD-18 T (Takara, Japan), and transformed into *Escherichia coli* DH5α (Takara, Japan). Subsequently, the products were directly sequenced by Quintara Biotechnology Company (Wuhan, China). For 3′-RACE, total RNA was subjected to reverse transcription with SMARTScribe™ Reverse Transcriptase and 3′CDS primer A (Clontech). The first-round PCR amplification was carried out with the primer 3′918–1 and UPM (Clontech). Then the second-round of PCR amplification was performed with the primer 3′918–2 and UPM (Clontech). The PCR products were gel-purified, and directly sequenced by Quintara Biotechnology Company (Wuhan, China).

To identify the homologs of *bmp2a*, *bmp2b*, *bmp4*, *bmp5*, *bmp7b*, *bmp8a*, *bmp14 and bmp16* in *M. amblycephala*, BLAST searches of the *M. amblycephala* genome (GeneBank accession number: SRP090157; Liu et al., 2017) were conducted using the *Danio rerio* reference transcripts for BMPs and partial cDNA sequences obtained from our previous transcriptomic data (GeneBank accession number: SRR233513). Then the cDNA sequences of *bmp2a*, *bmp2b*, *bmp4*, *bmp5*, *bmp7b*, *bmp8a*, *bmp14 and bmp16* were predicted, and subsequently complemented and validated by homologous cloning. Based on the sequences of BMPs in *Danio rerio* and partial cDNA sequences of these *bmp* genes, primers for homologous cloning were designed (Table S1). For isolating full-length clones, cDNA from total RNA extracted from the fin was used as template for PCR. The PCR products were gel-purified, ligated into the T/A cloning vector pMD-18T, and transformed into *Escherichia coli* DH5α. Subsequently, the products were directly sequenced by Quintara Biotechnology Company (Wuhan, China).