Temporal and Spatial Expression Patterns of Bone Morphogenetic Protein 3 in Developing Zebrafish

The Open Rheumatology Journal, 2014, *8*, 69-72

Temporal and Spatial Expression Patterns of Bone Morphogenetic Protein 3 in Developing Zebrafish

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Abstract: Bone morphogenetic proteins (BMPs) are important elements in bone biology. We herein report the expression profiles of zebrafish *bmp3* (*zbmp3*) as demonstrated by real-time PCR and *in situ* hybridization. The expression of *zbmp3* was highly detectable by real-time PCR from 1 day post-fertilization (1 dpf) to 2 weeks post-fertilization (2 wpf) and peaked at 1 wpf. For *in situ* hybridization experiments, *zbmp3* was expressed in the otic vesicle at 1 dpf, 2 dpf, 3 dpf, and 5 dpf. It was also expressed in the pharyngeal arches, including the opercle, branchiostegal ray, and pectoral fins, at 2 dpf. Our results suggest that *zbmp3* may play an important role in the skeletal biology of developing zebrafish.

Keywords: Bmp3, Expression patterns, Zebrafish.

INTRODUCTION

Bone morphogenetic proteins (BMPs) are members of the transforming growth factor-beta (TGF-beta) superfamily. Although most BMPs are positive regulators of bone biology, BMP3 is an antagonist of osteogenic BMPs [1]. The *BMP3* gene is highly conserved across species, suggesting that it is evolutionarily important for development in mammals [2]. A previous study uncovered that murine *Bmp3* was expressed in the cartilaginous cells of otic capsule sections using *in situ* hybridization [3]. However, as there have been few reports on the temporal and spatial expression profiles of *Bmp3* in whole animals, we examined the expression patterns of *bmp3* in developing vertebrates using a zebrafish model system.

MATERIALS AND METHODS

Extraction of Total RNA from Zebrafish Embryos and Cloning of Zebrafish *bmp3* (*zbmp3*)

Total RNA from zebrafish embryos and larvae was extracted at selected time points using an Isogen extraction kit (*Wako Co.*, Japan). Full-length *zbmp3* (GenBank accession no. NM_001077765) was cloned into a pCR-Blunt2-TOPO vector (*Invitrogen*, Japan) between EcoRI sites. The primers used to amplify the *zbmp3* full-length cDNA were 5'-ATGGATCGCTGTCAGCGCCTGTTTGTC CTCC-3' (forward) and 5'-TTACCGACAGGCGCAGGAG TCCACTGTCATG-3' (reverse). The PCR product size was 1359 bp.

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Real-Time Polymerase Chain Reaction (PCR)

The primers used to amplify the *zbmp3* cDNA for real-time PCR experiments were 5'-AAGGGCCATTTGGGA ACCAT-3' (forward) and 5'-TGTGGCTGCTGTTGTG AAGA-3' (reverse) The PCR product size was 130 bp. Real-time PCR using SSo Advanced SYBR Green supermix (*BioRad*, Japan) with 1-cell stage embryos (1 cell) and at 3 hours post-fertilization (hpf), 8-9 hpf, 1 day post-fertilization (dpf), 2 dpf, 3 dpf, 1 week post-fertilization (wpf), and 2 wpf were performed as described previously [4].

In Situ Hybridization

In situ hybridization was performed as earlier described [5] on 1, 2, 3, and 5 dpf embryos and larvae using full-length *zbmp3*. Digoxigenin-labeled RNA probes were generated using an Sp6 labeling kit (Roche, USA) according to the manufacturer's instructions. Signals were detected with an ALP-conjugated anti-digoxigenin antibody (Roche) and visualized using 4-nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-phosphate. The reaction was stopped by replacing the substrate with several rinses of PBT (PBS plus 0.1% Tween 20). Embryos were stored in the dark at 4°C in PBS containing 0.02% sodium azide until they were photographed.

RESULTS

As shown in Fig. (**1**), the real-time PCR expression level of *zbmp3* was very low at 1 cell, 3 hpf, and 8-9 hpf time points. However, *zbmp3* expression was dramatically increased from 1 dpf to 2 wpf and peaked at 1wpf.

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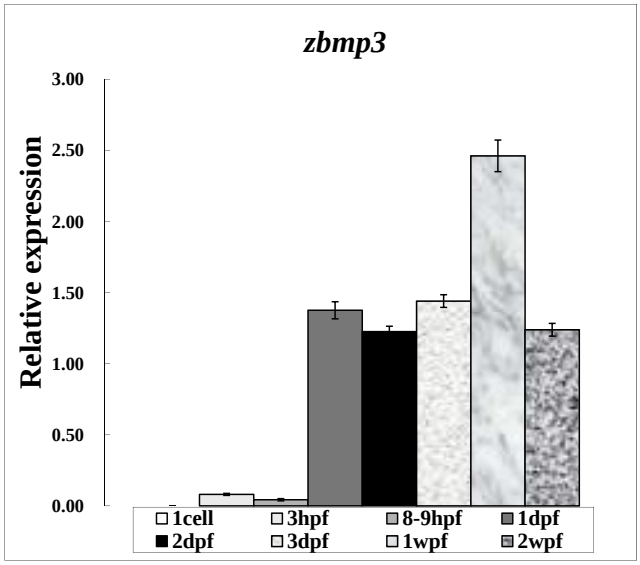

Fig. (1). Expression study of *zbmp3* by real-time PCR using samples from 1 cell, 3 hpf, 8-9 hpf, 1 dpf, 2 dpf, 3 dpf, 1 wpf, and 2 wpf time points.

For *in situ* hybridization experiments, *zbmp3* was not detectable before 1 dpf, but was specifically expressed in the otic vesicle at 1 dpf (Fig. **2a**; black arrows), 2 dpf (Fig. **2b**, **c**; black arrows), 3 dpf (Fig. **2d**, **e**; black arrows), and 5 dpf (Fig. **2f**, **g**; black arrows). In addition, as Cheah *et al.* have reported previously, *zbmp3* was also expressed in the opercle (op) and branchiostegal ray (bsr) (Fig. **2c**; black arrowhead) and pectoral fins (Fig. **2c**; white arrowhead) at 2 dpf. Each experiment was repeated at least twice. A sense (forward) probe of z*bmp3* was used as a control, for which no specific signal was detected.

DISCUSSION

We observed increased and localized expression of *zbmp3* at specific time points in the present zebrafish development model study. Our results suggest that bmp3 may be a key player in skeletogenesis in vertebrates.

Schoenebeck *et al.* have reported that *zbmp3* was expressed in the pharyngeal arches and pectoral fins of zebrafish at 48-72 hpf. However, *zbmp3* expression in the otic vesicle was not remarkable in their study as it was in ours (Fig. **2a-g**) [6]. The reason for this disparity is not clear.

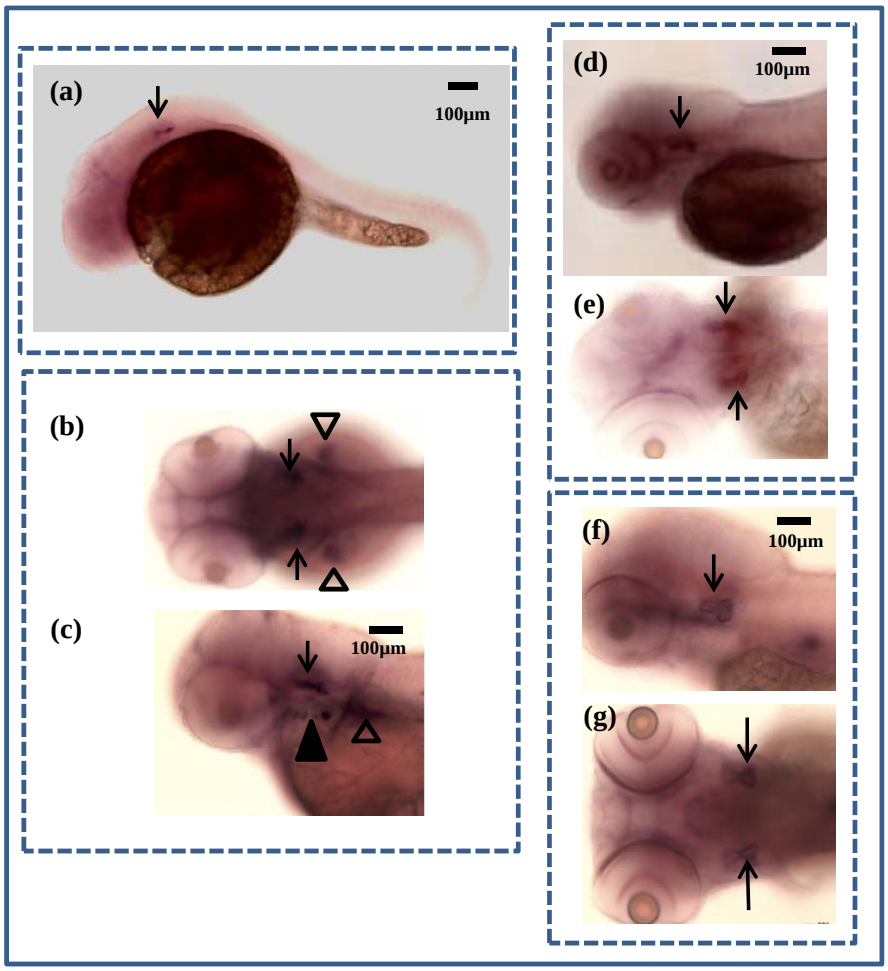

Fig. (2). *In situ* hybridization of *zbmp3* in developing zebrafish. (**a**) Lateral view of a 1 dpf zebrafish. Arrow indicates otic vesicle. (**b**) Dorsal view of a 2 dpf zebrafish. Arrows indicate otic vesicles and arrowheads show pectoral fins. (**c**) Lateral view of a 2 dpf zebrafish. Arrow indicates otic vesicle, black arrowhead shows opercle and branchiostegal ray, and white arrowhead shows pectoral fin. (**d**) Lateral view of a 3 dpf zebrafish. Arrow indicates otic vesicle. (**e**) Dorsal view of a 3 dpf zebrafish. Arrows indicate otic vesicles. (**f**) Lateral view of a 5 dpf zebrafish. Arrow indicates otic vesicle. (**g**) Dorsal view of a 5 dpf zebrafish. Arrows indicate otic vesicles.

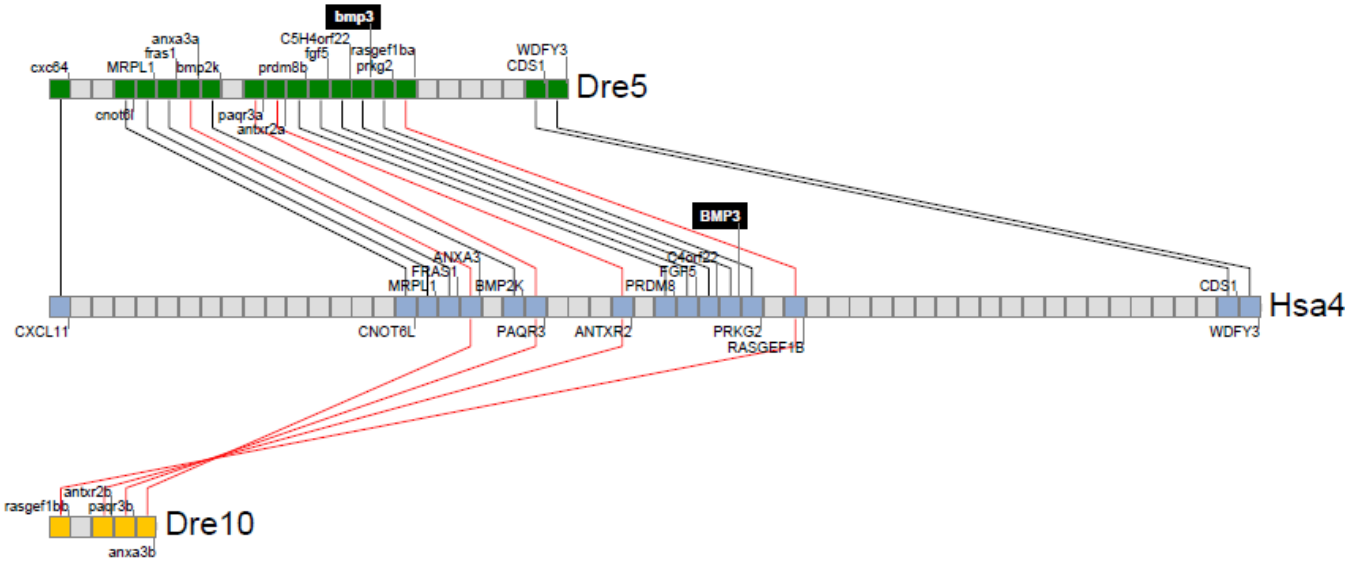

Fig. (3). Synteny analysis for *zbmp3* with human *BMP3*. Zebrafish and human *bmp3* genomic loci were analyzed and their neighbor genes were listed in order of occurrence in zebrafish and human genomes. Shared neighbor genes were linked between zebrafish and humans to reveal evolutionary conservation of this specifc genomic locus. The *bmp3* gene is indicated by a black background on zebrafish chromosome 5 and human chromosome 4.

Whereas Schoenebeck used an Oregon AB line [6], we adopted Cyprinidae, Rasborinae (Danio Rerio). Therefore, it is possible that a difference in genetic background may influence *zbmp3* expression patterns in zebrafish lines.

The vertebrate inner ear has two major functions: 1) maintenance of body balance and 2) auditory perception. The otic vesicle plays a role in the development of ear interaction with the hindbrain [7]. Since *zbmp3* was strongly expressed in the otic vesicle in zebrafish, it may influence the development of the zebrafish ear.

Runx2 is a transcriptional factor required for osteoblastic differentiation and the development of hypertrophic chondrocytes. In zebrafish, there are 2 orthologs of *Runx2*, *runx2a* and *runx2b* [8]. These genes were found to be expressed in the developing zebrafish skeleton, including the pharyngeal arch, op, and pectoral fins, which coincided with *zbmp3* expression patterns in the present study. These results suggest a functional overlap among *runx2a*, *runx2b,* and *bmp3* in zebrafish.

To our knowledge, there have been no reports on the relationship between BMP3 and rheumatologial diseases. However, other BMP family members, such as BMP4, BMP6, and Runx2, are possible biomarkers of bone metabolism in several forms of arthritis since their expression patterns are different in OA and RA groups from those in controls [9]. Based on our findings, further studies are warranted on the role of BMP3 in patients with rheumatic disease.

Gene duplication has been observed in the zebrafish genome due to an additional whole genome duplication event that is specific to the teleost fish lineage [10]. To study the conservation of *Bmp3* function in vertebrates, we lastly performed a synteny analysis for *zbmp3* with human *BMP3* as shown in Fig. (**3**). We found only one *zbmp3* gene, on chromosome 5, in the zebrafish genome, although a chromosomal segment duplicate on chromosome 10 that harbored a deleted *zbmp3* duplicate was detected as well. Thus, one *zbmp3* duplicate appears to have been lost in chromosome 10 during zebrafish genome evolution.

CONCLUSION

Our results suggest that *zbmp3* plays a role in the arch skeletal system in developing zebrafish. Functional analyses using genetically modified zebrafish are needed to better understand the *in vivo* biological functions of *zbmp3*. The relationship of BMP3 with OA and RA may also require further analysis.

AUTHOR CONTRIBUTIONS

Y.N. directed this study; M.I.A. and M.M. performed experiments and drafted the article; X.H., M.H., M.M., R.W. and H.K. contributed to conception and design, interpreted the data, and revised the article. All authors approved the final version.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

ACKNOWLEDGEMENTS

Declared none.

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Received: February 4, 2014 Revised: July 18, 2014 Accepted: July 19, 2014