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Characterization and embryonic expression of four amphioxus *Frizzled* genes with important functions during early embryogenesis

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ABSTRACT

The Wnt signaling pathway plays crucial roles in the embryonic patterning of all metazoans. Recent studies on *Wnt* genes in amphioxus have shed important insights into the evolution of the vertebrate *Wnt* gene family and their functions. Nevertheless, the potential roles of *Wnt* family receptors encoded by *Frizzled* (*Fz*) genes in amphioxus embryonic development remain to be investigated. In the present study, we identified four amphioxus *Fz* genes—*AmphiFz1/2/7*, *AmphiFz4*, *AmphiFz5/8,* and *AmphiFz9/10*—and analyzed their expression patterns during amphioxus embryogenesis. We found that these four *Fz* genes were maternally expressed and might be involved in early animal-vegetal axis establishment. The *AmphiFz1/2/7* transcripts were detected in the central dorsal neural plate, mesoderm, the Hatschek's pit, and rim of the mouth, whereas those of *AmphiFz4* were detected in the mesoderm, pharyngeal endoderm, and entire gut region. *AmphiFz5/8* was exclusively expressed in the anterior-most region, whereas *AmphiFz9/10* was expressed in the neural plate, somites, and tail bud. The dynamic and diverse expression patterns of amphioxus *Fz* genes suggest that these genes are not only associated with early embryonic axis establishment but also are involved in the development of several organs in amphioxus.

The Wnt signaling pathway has been extensively studied for its critical roles in a myriad of developmental events, such as cell differentiation, morphogenesis, tissue homeostasis (Clevers and Nusse, 2012; Grigoryan et al., 2008), and embryonic patterning, as well as body plan formation (Petersen and Reddien, 2009; Rao and Kuhl, 2010; van Amerongen and Nusse, 2009). In this pathway, Wnt ligands interact with their receptor *Frizzled* (*Fz*) proteins at the cell surface, typically initiating a signaling cascade that shuts cytoplasmic β-catenin away from degradation and allows it to combine with T-cell factor (TCF)/lymphocyte enhancer factor (LEF) to modulate gene transcription in the nucleus (Buechling and Boutros, 2011). Recent sequencing analysis of metazoan genomes has revealed that *Wnt* and *Fz* genes appear in the last common ancestor of demosponge and eumetazoans (Adamska et al., 2010). In fact, the number of *Wnt* genes varies across living metazoans from 5 to 27 approximately, but its receptors seem to maintain four members consistently except the vertebrate lineage (Holstein, 2012; van Amerongen and Nusse, 2009). For example, only 7 *Wnt* and 4 *Fz* genes were found in *Drosophila*, 11 *Wnt* and 4 *Fz* genes were found in sea urchin, *Strongylocentrotus purpuratus* (Croce et al., 2006), and 10 *Wnt* and 4 *Fz* genes were found in urochordate, *Ciona intestinalis* (Delsuc et al., 2006). However, at least 19 *Wnt* and 10 *Fz* genes were revealed in vertebrates. Thus, the relatively large number of *Wnt* and *Fz* genes in vertebrate genomes raises the question about their implications in the evolution of vertebrate-specific morphological characteristics. Amphioxus is considered a good system to investigate this question owing to its basal position within the chordates (Delsuc et al., 2006), pre-duplicated genomic organization (Putnam et al., 2008; Yu and Holland, 2009b), and embryonic body plan similar to that of vertebrates in having characteristics such as a nerve cord, notochord, pharyngeal gill slits, segmental muscles and so on (Bertrand and Escriva, 2011; Holland et al., 2004).

The *Fz* gene family of vertebrates has been suggested to be involved in early embryonic axis formation, mesoderm, and neural crest induction, development of the central nervous system, and other multiple organogenesis processes (Kemp et al., 2007; Nikaido et al., 2013; Verkade and Heath, 2008). Here, to investigate the functional evolution of *Fz* genes in early embryogenesis during the invertebrate-to-vertebrate transition, we characterized the complete set of *AmphiFz* gene family members in the cephalochordate amphioxus and analyzed their spatial–temporal expression patterns to unravel the functional roles of this gene family during amphioxus early embryogenesis.

1. Results

1.1. Characterization of AmphiFz genes in amphioxus

In the present investigation, our *in silico* analyses revealed gene fragments encoding four candidate *Fz* genes—*AmphiFz1/2/7*,

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AmphiFz4, *AmphiFz5/8,* and *AmphiFz9/10*—from the genome of Chinese amphioxus *Branchiostoma belcheri* (http://mosas.sysu.edu.cn/genome). The entire coding sequences of these four *AmphiFz* genes have been deposited in GenBank under the accession numbers KC690271 (*AmphiFz1/2/7*), KC690272 (*AmphiFz4*), KC690273 (*AmphiFz5/8*), and KC690274 (*AmphiFz9/10*). Their deduced complete open reading frames (ORFs) are predicted to be 1680, 1626, 1680, and 1695 bp, respectively, and they encode proteins comprising 559, 541, 559, and 564 amino acids, respectively. Each *AmphiFz* protein typically contains nine highly conserved domains—one extracellular cysteine-rich domain (CRD), including ten conserved cysteine residues, seven trans-membrane domains (TMD), and one conserved cytoplasmic motif [K(Lys)-T(Thr)-X-X-X-W(Trp)] immediately adjacent to the TMD7 (Fig. 1). These conserved domains are also found in the *Fz* homologs of vertebrates (Schulte and Bryja, 2007). Our phylogenetic analyses further divided the deuterostome *Fz* family members into five subfamilies, namely *Fz1/2/7*, *Fz3/6*, *Fz4*, *Fz5/8,* and *Fz9/10*. Of these five identified subfamilies, all homologous genes (*AmphiFzs*), except *Fz3/6*, were found in the amphioxus genome, and each *AmphiFz* represented the pro-ortholog of one or several vertebrate paralog (Fig. 2).

1.2. Temporal expression patterns of AmphiFz genes during embryogenesis

Real-time reverse transcription-polymerase chain reaction (RT-qPCR) analyses showed that all of the four *AmphiFz* genes were expressed in the unfertilized egg. *AmphiFz1/2/7* maintained a constant maternal expression level from the unfertilized egg stage to the late blastula stage and showed a significantly increased expression level at the mid-gastrula stage, indicating the start of zygotic transcription of this gene. After gastrula, the amount of *AmphiFz1/2/7* mRNA began to decrease gradually until the lowest level at the early larva stage (Fig. 3A). In contrast to *AmphiFz1/2/7*, *AmphiFz4* started its zygotic expression at the four-cell stage because the copy number of its transcripts increased evidently (Fig. 3B). This gene expression continuously increased until the highest level at the mid-gastrula stage, and then the high expression level was maintained until the early larva stage. On the other hand, the zygotic expression of *AmphiFz5/8* started at the late blastula stage and reached the highest level at the mid-gastrula stage (Fig. 3C). Immediately after this stage, the expression decreased significantly and was maintained at a moderate expression level until the early larva stage. Finally, the expression profile

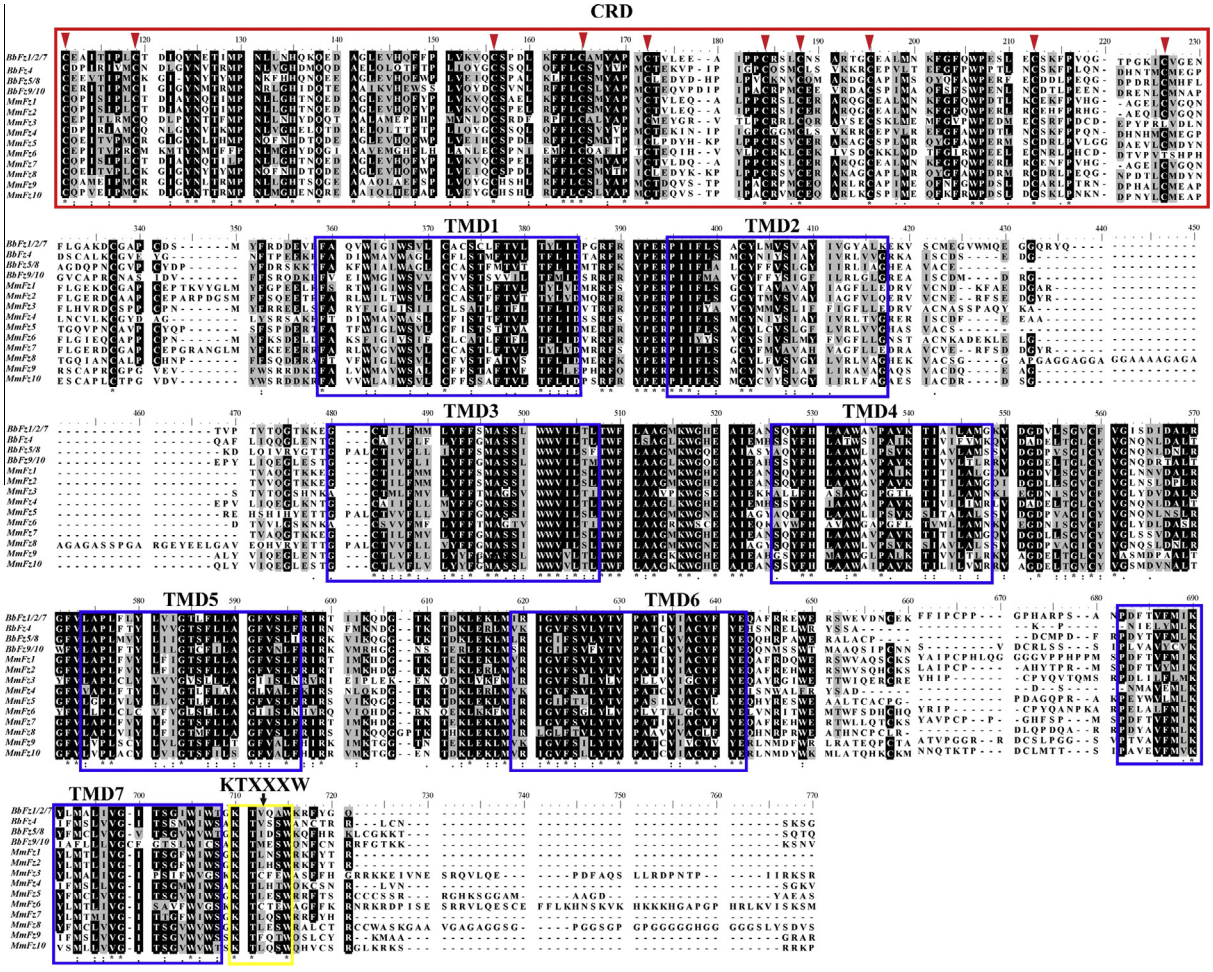

Fig. 1. Multiple sequence alignment of conserved region in *Fz* proteins of amphioxus (*Branchiostoma belcheri*) and mouse (*Mus musculus*). The protein names are abbreviated as follows: BbFz1/2/7, BbFz4, BbFz5/8, BbFz9/10, MmFz1 (NP_067432), MmFz2 (NP_065256), MmFz3 (NP_067433), MmFz4 (NP_032081), MmFz5 (NP_073558), MmFz6 (NP_001155966), MmFz7 (NP_032083), MmFz8 (NP_032084), MmFz9 (NP_034376) and MmFz10 (NP_780493). The highly or moderately conserved amino-acids are respectively shaded in black or gray with 60% threshold. The cysteine-rich domain (CRD), seven trans-membrane domains (TMDs) and a conserved cytoplasmic motif [K (Lys)-T (Thr)-X-X-X-W (Trp)] are respectively indicated by red, blue and yellow rectangle boxes, and ten conserved cysteines are indicated by red arrowheads.