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Dorsal fin development in flounder, *Paralichthys olivaceus*: Bud formation and its cellular origin

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The development of the median fin has not been investigated extensively in teleosts, although in other fishes it has been proposed that it involves the same genetic programs operating in the paired appendages. Adult median fins develop from the larval bud; therefore an investigation of fin bud formation and its cellular origin is essential to understanding the maturation mechanisms. In *Paralichthys olivaceus*, skeletogenesis proceeds from an anterior to posterior direction providing a good opportunity to study the formation of dorsal fin bud. An apical ectodermal ridge appeared at the basal stratum of the presumptive dorsal fin was first observed at 3 days post hatching. Then the apical ectodermal fold formed as the bud outgrew in 6 days post-hatch larvae. The bud continued to grow, breaking through the dorsal fin fold in 9 days post-hatch larvae. At 13 days post-hatch, the bud grew beyond the edge of the fin fold and formed into the four future rays. Molecular markers of cell type showed the existence of neural crest cells, scleroblasts and sclerotomes in the dorsal fin bud. The earliest gene expression in the dorsal fin bud was *Hoxd10* at 3 days post-hatch larvae, then *Hoxd9*, *Hoxd11* and *Hoxd12*. This indicates *Hoxd10* might be a candidate molecular marker of the bud formation site. Some key molecular markers for paired appendage development, such as *FGF8*, *Wnt7*, and *Shh* were expressed at the apical ectodermal ridge and later the apical ectodermal fold. Moreover, the form of the dorsal fin bud could be inhibited by Hh pathway inhibitor, further indicating that common basic molecular mechanisms might be utilized by median fins.

1. Introduction

Median fins, which include dorsal, caudal and annular fins, lie along the anterior-posterior body axis. In extant vertebrate species, representatives of these are found in both the Agnatha (lampreys and hagfish) and Gnathostomata (Coates, 1994; Donoghue et al., 2000). Unlike paired limb appendages or pectoral fins, median fin development has not yet been extensively studied. The median fins are positioned within an initially continuous median larval fin fold (Mabee et al., 2002; Suzuki et al., 2003; van Eeden et al., 1996). The development of the adult median fins is separated into three distinct phases. In phase 1, a continuous fin fold is fully developed along the dorsal and ventral midline. In Phase 2, each of the presumptive median fins is positioned along the anterior to posterior axis of the fin fold. As the fins develop, the fin fold degenerates. In Phase 3, the radials and fin rays differentiate (Mabee et al., 2002). In zebrafish, for example, the median fin fold develops during embryogenesis. During the larval stages the fin fold is replaced by four different fins, the unpaired anal, dorsal and tail fins. In zebrafish ranging between 5.2 and 5.5 mm in standard length, the bud of the anal fin is first observed as a bulge resulting from the condensation of mesenchyme. Later in development, at the 5.5–5.8 mm size, the bud of the dorsal fin can be observed (Parichy et al., 2009).

The appearance of the fin bud as described in phase 2 marks the position of adult fin formation, and is the base for subsequent fin rays differentiation (Mabee et al., 2002). However, since at least 118 mutations affecting larval fin formation have been identified in

Abbreviations: AEF, apical ectodermal fold; AER, apical ectodermal ridge; *Col10a1*, collagen type l0 alpha 1; DIG, digoxigenin; *FGF8*, Fibroblast growth factor 8; Hh, hedgehog; *Hox*, homeobox gene; M-MLV, moloney murine leukemia virus; MS 222, tricaine methanesulfonate; *Msx2*, muscle segment homeobox 2; PBST, phosphate buffered saline tween; PFA, paraformaldehyde; Scleraxis, sclerotome-related helix-loop-helix type transcription factor; Shh, Sonic hedgehog; *Wnt7*, wingless-type 7.

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zebrafish, and most mutants survive to adulthood and form a surprisingly normal complement of adult fins, the differentiation processes forming the embryonic fin fold and adult fins are probably largely independent (van Eeden et al., 1996). Therefore, in order to better understand adult median fin development, further attention should be directed at bud formation.

The bud formation for the adult median fin in the cat shark *Scyliorhinus canicula* starts from an apical ectodermal ridge (AER)-like structure beneath the fin fold (Freitas et al., 2006), as in the AER seen at the distal margin of the buds in the paired fins of teleosts (Saunders, 1948; Grandel and Schulte-Merker, 1998). The AER then becomes an apical ectodermal fold (AEF) (Akimenko et al., 1994, 1995; Monnot et al., 1999; Abe et al., 2007; Grandel et al., 2000). In the cat shark, it has been shown that median fins arise predominantly from somatic (paraxial) mesoderm, whereas paired appendages develop from lateral plate mesoderm. Moreover, it has been demonstrated that shark median fin development involves the same genetic programs that operate in paired appendages (Freitas et al., 2006). In zebrafish, it has been proposed that the fin fold mesenchyme is derived from neural crest cells (Smith et al., 1994), but the recent research suggested that it is not derived from the trunk neural crest (Lee et al., 2013a; 2013b). In teleosts, bud formation and its cellular origin have not been extensively investigated yet. Since the adult median fins in major living fishes are positioned within larval fin folds, the formation of buds is not easily observed during adult median fin development (Mabee et al., 2002; Suzuki et al., 2003; van Eeden et al., 1996).

In some flatfish species, skeletogenesis proceeds from an anterior to posterior direction providing a good opportunity to study the formation of dorsal fin bud (Inui and Miwa, 1985; de Jesus et al., 1990). The differentiation of chondrocytes and scleroblasts during dorsal fin skeletogenesis in larval flounder *Paralichthys olivaceus* has been studied (Suzuki et al., 2003). However, the formation of the dorsal fin bud has not been studied during post-embryo development of *P. olivaceus*. Based on observations of the developmental process of the dorsal fin bud, we investigated its cellular origins using molecular markers for different cell types. Following the previously described mechanism on the formation of limb buds (Freitas et al., 2006; Kawakami et al., 2003; Gibert et al., 2006; Marí-Beffa and Murciano, 2010), the expression of genes including Hox, Wnt family and Shh were investigated. The aim of the work was to investigate the positioning and induction on the fin bud during dorsal fin development in *P. olivaceus*.

2. Results

2.1. Formation of dorsal fin bud and skeletogenesis

A continuous fin fold was developed along the dorsal and ventral midline in 3 days post-hatch larvae (Fig. 1A1). In this stage, the AER was shown as a very small fold at its basal stratum of the presumptive dorsal fin (Fig. 1A6,A11). The bud outgrew noticeably in the larval dorsal fin fold close to the head in 6 days post-hatch larvae. Formation of an AEF was obvious (Fig. 1A7,A12). The bud became larger and its corresponding site of fin fold became a nick in 9 days post-hatch larvae (Fig. 1A8). The base of the bud also became larger and began to form over several sections, which later differentiated into the pterygiophores, including proximal, medial and distal radials (Fig. 1A13). At 13 days post-hatch, the top of the bud had grown beyond the edge of the fin fold and formed into what would later become four rays (Fig. 1A9). The four sections of the future pterygiophores at the extending base of the bud had also developed noticeably (Fig. 1A14). In larvae up to and including 16 days post-hatch, the rays and pterygiophores could be stained by Alcian blue (Fig. 1B1,B3). Five pterygiophores and five rays could be seen in larvae at 16 days post-hatch (Fig. 1A10). The numbers of pterygiophores and rays had increased at 19 days post-hatch larvae and rays began to ossify into lepidotrichia (staining as red in Fig. 1B2, B4).

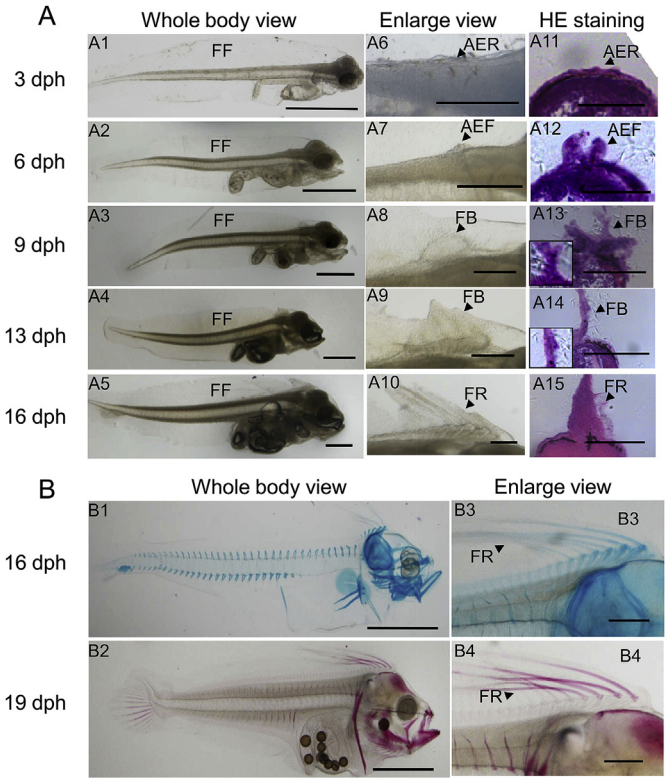

Fig. 1. The formation process of dorsal fin bud and skeletogenesis in *P. olivaceus*. A. the whole body view, enlarged view and HE staining of 3, 6, 9, 13, and16 days post-hatch larvae. Enlarged view of fin bud is shown in the inset of the panels, A13 and A14. Bars: 500 μm in panels A1- A5, 200 μm in panels A5 - A10, 20 μm in panels A11-A12, and 50 μm in panels A13-A14. AER (apical ectodermal ridge), AEF (apical ectodermal fold), FB (fin bud), FF (fin fold), FR (fin ray). B. Cartilage staining (blue) of 16 days post-hatch larvae and bone staining (red) of 19 days post-hatch larvae. Bars: 1 mm in panels B1-B2, and 200 μm in panels B3-B4. Arrow indicates the fin ray.

2.2. Cellular origins of the dorsal fin bud

The formation process of the dorsal fin bud in *P. olivaceus* is similar to that observed in the process of AEF formation during the development of paired fins of teleosts. In order to understand the cellular origins of the bud of the dorsal fin, the expression of *Scleraxis* (sclerotome-related helix-loop-helix type transcription factor), a marker of sclerotomal cells, was examined in *P. olivaceus*. *Scleraxis* was expressed in the AER at the presumptive dorsal fin site in 3 days post-hatch larvae (Fig. 2A1). However, it was expressed weakly in the later bud formation and thereafter skeletogenesis (Fig. 2A2–A7).

To gain some insight into the contribution of neural crest cells in dorsal fin bud formation, we further investigated the expression pattern of three genes, *Slug, Hnk-1 and Msx2*, which are known to serve as markers of neural crest cells. These three genes were expressed at the AER in 3 days post-hatch larvae of *P. olivaceus* and persisted during the processes of dorsal fin bud formation and later skeletogenesis (Fig. 2B–D). Compared with *Hnk-1* and *Msx2*, the expression of *Slug* was maintained at a higher level during the whole process of fin development (Fig. 2B). The another difference with *Hnk-1* and *Slug* is that *Msx2* expressed weakly in fin fold of 6, 9,