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Development of the lateral line canal system through a bone remodeling process in zebrafish

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ABSTRACT

The lateral line system of teleost fish is composed of mechanosensory receptors (neuromasts), comprising superficial receptors and others embedded in canals running under the skin. Canal diameter and size of the canal neuromasts are correlated with increasing body size, thus providing a very simple system to investigate mechanisms underlying the coordination between organ growth and body size. Here, we examine the development of the trunk lateral line canal system in zebrafish. We demonstrated that trunk canals originate from scales through a bone remodeling process, which we suggest is essential for the normal growth of canals and canal neuromasts. Moreover, we found that lateral line cells are required for the formation of canals, suggesting the existence of mutual interactions between the sensory system and surrounding connective tissues.

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Introduction

The lateral line is composed of mechanoreceptors called neuromasts, which are distributed over the body of fish and amphibians. Neuromasts comprise a core of sensory hair cells surrounded by nonsensory support cells. Each neuromast is innervated by a branch of the lateral line nerve. Recent progress has enhanced the understanding of how superficial neuromasts form in the epidermis of embryos and larvae (reviewed by Ghysen and Dambly-Chaudière (2007)). During embryonic development, the multiple placode-derived lateral line primordia migrate across the epidermis to deposit neuromast precursors or proneuromasts (Gompel et al., 2001), which then integrate into the epidermal layer and differentiate into neuromasts (Sapède et al., 2002). During postembryonic development, some of the embryonic neuromasts remain on the body surface (superficial neuromasts), while the remainder become embedded in the body (canal neuromasts) (Webb and Shirey, 2003).

Superficial neuromasts maintain the same size throughout fish life, which during early embryogenesis, is regulated by the balance between growth-promoting Wnt signaling activity and Wnt-inhibiting Dkk activity produced by the sensory hair cells (Wada et al., 2013). New superficial neuromasts form through local proliferation of a strand of interneuromast cells that connects the neuromasts to each other (Grant et al., 2005; López-Schier and Hudspeth, 2005), or through a budding process whereby each embryonic neuromast gives rise to a number of accessory neuromasts (Ledent, 2002; Wada et al., 2010).

The lateral line canals are a prominent feature of the lateral line system, and the canal neuromasts are found within the canals, in vast majority of fishes. Canal neuromasts are thought to function as acceleration rather than velocity detectors (reviewed by Denton and Gray (1989)), and also differ from superficial neuromasts developmentally in that they increase in size throughout adult life, rather than keeping a constant size and increasing in number (Janssen et al., 1987; Münz, 1989; Webb and Shirey, 2003). This difference is all the more surprising considering that canal neuromasts are born as superficial neuromasts (Webb and Shirey, 2003).

Canal neuromasts on the trunk region are embedded in ossified canals made by specialized scales (lateral line scales). These scales are often distinguishable to the naked eye, and form the so-called "lateral line" that extends along the flank of most teleost fishes. Lateral line scales can be regenerated after removal (Mori, 1931a; Bailey, 1937), and the canal reforms across a normal scale when it is transplanted to the lateral line (Mori, 1931b; Bailey, 1937). Moreover, the lateral line cells can induce canal bone morphogenesis (Merrilees, 1975). These results suggest a role of the lateral line in regulating bone development.

To better understand the rules and mechanisms that govern the formation of canal neuromasts, we have examined the formation of the trunk canal neuromasts in zebrafish using molecular

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markers and genetic tools. Zebrafish, unlike goldfish and most teleost species, have only a few tubed lateral line scales along the anterior-most trunk region. Herein, we suggest that trunk canals develop and are maintained through a bone remodeling process, which requires both bone-forming cells (osteoblasts) and bone-resorption cells (osteoclasts). Analyses of mutant fish, together with ablation experiments, suggest that both the presence of neuromasts and the bone remodeling process could be important for normal development of the trunk canals.

Materials and methods

Fish strains

Zebrafish *cldnb:gfp* (highlights neuromast cells and the epidermis, Haas and Gilmour, 2006), *atoh1a:rfp* (highlights neuromast hair cells, Wada et al., 2010), Gal4 enhancer trap lines SAGFF(LF)228A (highlights neuromast hair cells and osteoblasts, Supplemental Fig. S2, Kawakami et al., 2010) and gSAzGFF15A (highlights neuromast hair cells, Supplemental Fig. S2, Kawakami et al., 2010), and *c-fms/panther*^{j4e1} (Parichy et al., 2000) mutant fish were used. The zebrafish strains, TAB (in the AB genetic background) and UT (in the *leopard*^{t1} genetic background) were maintained in our laboratory. Standard length (SL) of each fish was measured. All experimental animal care was in accordance with institutional and national guidelines and regulations.

Tartrate-resistant acid phosphatase (TRAP) staining

We performed the Azodye method as described (Chatani et al., 2011) to detect tartrate-resistant acid phosphatase (TRAP) enzymatic activity. The specimens were incubated in a mixture of 0.5 mM naphthol AS-MX phosphate (Wako), 1.5 mM Fast Red Violet LB salt (Sigma), and 50 mM L(+)-tartrate in 0.2 M acetic buffer (pH 5.0) at 37 °C for the appropriate reaction time.

in situ hybridization

in situ hybridization using RNA probes for *sp7* (DeLaurier et al., 2010) and *ctsk* (Chatani et al., 2011) was performed as described (Westerfield, 2007). Images were captured using a differential interference contrast (DIC) microscope (Olympus BX-51WI) equipped with a CCD camera (Zeiss AxioCam MRc).

Neuromast and skeletal staining

Neuromasts were labeled by incubating live fish in 2 μm/ml 4-(4-diethylaminostyryl)-N-methylpyridinium iodide (DiAsp, Sigma) as described previously (Collazo et al., 1994). Labeling neuromasts with alkaline phosphatase was performed as described (Villablanca et al., 2006). Bone was stained with Calcein or Alizarin red (Wako Pure Chemical Industries) as described (Kimmel et al., 2003; Javidan and Schilling, 2004). Fish were anesthetized with Tricain (3-amino benzoic acid ethylester, Sigma) before observation. Images were captured using a fluorescence microscope (Leica M165FC) equipped with a CCD camera (Nikon DXM1200F), or by confocal microscopy (Zeiss LSM700).

Measurement of canal diameter and neuromast size

To visualize trunk canals and to measure canal diameter, an aliquot of black ink (100% Sumi Ink, Kuretake) or red ink (0.15% Cochineal Red A, Kyoritsu Foods) was dropped onto the anesthetized fish, and then excess ink was removed using a piece of filter paper placed on the skin surface. The lateral line scales were counted in UT ($n=60$, both sides from 30 fish, 28.0–34.0 mm SL) and TAB strains ($n=60$, both sides from 30 fish, 27.0–32.0 mm SL, Supplemental Fig. S1). To quantify canal diameter in relation to body size, five fish (UT strain, 15.0–28.0 mm SL) were analyzed (Fig. 3J). To compare canal diameters, *c-fms/panther* fish and heterozygous siblings (28.0–32.0 mm SL, *c-fms* −/−, $n=11$; *c-fms* +/−, $n=14$) were used (Fig. 5K), and to quantify neuromast area in relation to body size, the area occupied by DiAsp-positive hair cells was measured in the UT strain (20.0–34.0 mm SL, $n=6$) using the "Find Edges" and "Measure" functions in ImageJ software (http://rsb.info.nih.gov/ij/) (Fig. 3K). Since hair cells extend to the edges of canal neuromasts in zebrafish (Webb and Shirey, 2003), the area stained with DiAsp provides an accurate reflection of neuromast size. Neuromast areas were compared using *c-fms/panther* fish and heterozygous siblings (larval fish: 8.5–11.0 mm SL, *c-fms* −/−, $n=6$; *c-fms* +/−, $n=5$; adults: 28.0–32.0 mm SL, *c-fms* −/−, $n=6$; *c-fms* +/−, $n=9$) (Fig. 5K). In all experiments, 2–8 canal neuromasts per fish were analyzed.

Ablation experiments

Scale removal and transplantation was carried out using fine forceps as described previously (Mori, 1931a,b; Bailey, 1937); 46 lateral line scales were removed from 23 fish (Fig. 7A–J; $n=46$, UT strain, 24.0–28.0 mm SL) and analyzed by Calcein staining at 2 days ($n=6$), 3 days ($n=26$), 5 days ($n=10$), and 3 months ($n=4$) after removal. Fish were also fixed at 5 days ($n=10$) or 3 months ($n=4$) after scale removal and subjected to Alizarin red staining. Eight lateral line scales were removed from 4 *c-fms/panther* fish ($n=8$, 28.0–32.0 mm SL) and stained 5 days after removal (Fig. 8J–L). For the scale transplantation experiments (Fig. 7K–N), one dorsal scale and one lateral line scale were removed, and the dorsal scale was inserted into the place from where the lateral line scale had just been removed, as described previously (Shinya and Sakai, 2011). Since dorsal scales contain melanophores and lateral line scales are nonpigmented, we could assess whether the transplanted scale is retained by the presence of pigment cells (Shinya and Sakai, 2011, Supplemental Fig. S3). Twelve fish (UT strain, 24.0–28.0 mm SL) underwent the transplantation procedure, which was successful in six fish (the transplanted scales dropped off in three fish, and the remaining three fish died), and were analyzed by Calcein and Alizarin red staining at three months after transplantation. To remove the epidermal tissues (Fig. 8), *cldn:gfp* fish were used to visualize the canal epidermis. Two consecutive canal scales were removed, and then the epidermal tissues remaining on the body were carefully removed using a sterile needle (0.45 mm diameter, Terumo, Fig. 8A). Of 16 fish (*cldn:gfp*, 24.0–28.0 mm SL) undergoing the procedure, 3 died and 1 fish did not regenerate scales; finally, 12 fish were imaged at 10 days ($n=8$) or 1 month ($n=4$) after the operation, and then subjected to Alizarin red staining. As controls, sham ablations (removal of two consecutive scales) were performed on the opposite side of the body ($n=10$, Fig. 8F).

Results

Structure of the trunk lateral line canals

The lateral line canal system has been described in various teleost species. In zebrafish, a comprehensive study analyzed development of the cranial canal system (Webb and Shirey, 2003). However, previous studies mention little about the trunk canal system. The trunk canal of adult zebrafish is composed of a row of specialized scales as observed in other species (e.g., Wonsettler and Webb (1997), Fig. 1A), with each lateral line scale

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