Evolution of Developmental Control Mechanisms

Allometric growth of the trunk leads to the rostral shift of the pelvic fin in teleost fishes

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Abstract

The pelvic fin position among teleost fishes has shifted rostrally during evolution, resulting in diversification of both behavior and habitat. We explored the developmental basis for the rostral shift in pelvic fin position in teleost fishes using zebrafish (abdominal pelvic fins) and Nile tilapia (thoracic pelvic fins). Cell fate mapping experiments revealed that changes in the distribution of lateral plate mesodermal cells accompany the trunk–tail protrusion. Presumptive pelvic fin cells are originally located at the body wall adjacent to the anterior limit of hoxc10a expression in the spinal cord, and their position shifts rostrally as the trunk grows. We then showed that the differences in pelvic fin position between zebrafish and Nile tilapia were not due to changes in expression or function of gdf11. We also found that hox-independent motoneurons located adjacent to the pelvic fins innervate into the pelvic musculature. Our results suggest that there is a common mechanism among teleosts and tetrapods that controls paired appendage positioning via gdf11, but in teleost fishes the position of prospective pelvic fin cells on the yolk surface shifts as the trunk grows. In addition, teleost motoneurons, which lack lateral motor columns, innervate the pelvic fins in a manner independent of the rostral–caudal patterns of hox expression in the spinal cord.

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Introduction

Two sets of paired appendages are common for gnathostomes, including cartilaginous fishes, actinopterygians and sarcopterygians. The anterior paired appendages (forelimbs/pectoral fins) are usually located at the anterior end of the trunk, whereas the posterior paired appendages (hindlimbs/pelvic fins) tend to be located at the posterior end, lateral to the cloaca. Pelvic fins of primitive actinopterygians (ray-finned fishes), including bichirs, sturgeons, gars and bowfins, also arise just in front of the anus near the posterior end of the abdominal cavity (Rosen, 1982). During teleost fish evolution, the position of the pelvic fins has shifted from near the anus to an insertion directly under the pectoral fin (thoracic level), and even farther forward (jugular level) in some modern teleosts (Nelson, 1994). The positional shift of the pelvic fins seems to have diversified locomotion and feeding behavior (Harris, 1936, 1938), thereby expanding the types of habitats occupied by teleosts. Fishes with basal fin morphologies often have extremely limited pelvic fin function. Whereas, in more derived fishes, the pelvic fins have a trimming function to reduce pitching and upward body displacement during braking (Harris, 1936, 1938). In some fast-swimming percoid fish groups, the pectoral fins are used almost entirely as brakes and the pelvic fins completely control rising, diving and tilting movements (Harris, 1936, 1938). The roles of the pelvic fins have been elucidated in fish in which the pectoral or pelvic fins have been amputated (Harris, 1938).

During teleost embryogenesis, pectoral fin buds appear within a few days post-fertilization (dpf), whereas pelvic fin buds develop much later (Grandel and Schulte-Merker, 1998; Fujimura and Okada, 2007). In zebrafish embryos, pectoral fin buds appear from the very thin layers of somatopleural lateral plate mesoderm subsequent to the protrusion of the trunk–tail from the yolk at 28 h post-fertilization (hpf), whereas initiation of pelvic fin buds is associated with metamorphosis from larval to juvenile forms, including formation of fin rays in dorsal, tail and anal fins at about 3 weeks post-fertilization (wpf) (Grandel and Schulte-Merker, 1998). Similarly, in Nile tilapia, pectoral fin buds appear at about 67 hpf after the trunk–tail protrusion, whereas pelvic fin buds appear on the yolk surface at 9 dpf (Fujimura and Okada, 2007).

There is substantial evidence that hox expression in the spinal cord or in somitic mesoderm specifies the axial pattern (Gruss and Kessel, 1991), and hox expression patterns in the lateral plate mesoderm
similarly may specify the position along the anterior–posterior axis to give forelimbs, interlimbs and hindlimbs (Cohn et al., 1997). Caudal displacement of the hindlimbs has been reported in mice lacking Gdf11 function (McPherron et al., 1999), whereas ectopic expression of Gdf11 in chick spinal cord causes the rostral displacement of the limb buds (Liu, 2006). These changes in limb position are accompanied by changes in hox expression and in the positions of motoneuron columns, pools and nerve projections. Motoneurons innervating the limb musculature, called the lateral motor column (LMC), are present only at cervical/brachial and lumbar levels. LMC identity in opposite forelimbs (brachial) and hindlimbs (lumbar) is defined by expression of Hox6 and Hox10 paralogs (Dasen et al., 2003; Shah et al., 2004; Wu et al., 2008). In tetrapods, the position of the forelimb is also associated with the anterior border of Hox6 expression in the paraxial mesoderm (Burke et al., 1995). Rostral shift of the shoulder girdle has also been observed in mice lacking Hox65 (Rancourt et al., 1995).

Here, we investigated the developmental basis for the rostral shift of pelvic fin position by examining the emergence of the pelvic fin during development of two teleost fishes—zebrafish and Nile tilapia. Our results show that allometric trunk–tail protrusion during development of teleost fishes causes the rostral shift of pelvic fins and that the position of presumptive pelvic fin cells, originally located at lumbar levels, can be influenced by gdf11 levels, as in tetrapods. In addition, we show that motoneurons residing above the pelvic fins, but not at the anterior border of hoxc10a expression in the spinal cord, innervate the pelvic fins teleost fishes.

Materials and methods

Fish embryos

Wild-type zebrafish (TL and Tu), Danio rerio, and Nile tilapia, Oreochromis niloticus, were reared as described (Kimmel et al., 1995; Fujimura and Okada, 2007).

Scanning electron microscopy

Scanning electron microscopy was performed as described (Cole et al., 2003).

Observation of embryos

Specimens were sacrificed or anesthetized using 0.02% MS-222 (Sigma) and mounted on a depression glass slide in 3% methylcellulose (Sigma) to allow positioning. To better visualize larvae, we stained the fixed embryos with Mayer’s hematoxylin solution (Wako) for 5–10 min and washed with deionized water several times as described (Fujimura and Okada, 2007). Total length of each fish was taken from the anterior tip of the lower jaw to the posterior end of the caudal fin via the urostyle.

Dil and quantum dots application to track cells

Dil application was performed as described with slight modifications (Karlstrom et al., 1996; Aizawa et al., 2007). Briefly, embryos were embedded in 1.5–2.5% low-melting-point agarose and the overlying agarose was removed to expose the desired area. Small amounts of Dil (5 mg/ml in dimethylformamide; Invitrogen Molecular Probes) or Qtracker reagents (Qtracker Cell Labeling Kits, Q5041MP, Invitrogen Molecular Probes) were injected via microelectrodes into the mesoderm using a microinjector (Narisihge). The Qtracker Cell Labeling Kits are designed to deliver Qdot nanocrystals into the cytoplasm. After injection, all agarose was removed, and the embryos were returned to fish water. In each embryo, the locations of tracer were photographed at various time points using a fluorescence microscope. Some embryos were fixed after a 2-hour incubation for anti-i-β-catenin antibody staining and observed under an LSM510 META laser confocal microscope (Carl Zeiss).

Whole-mount in situ hybridization

Whole-mount in situ hybridization was performed as described (Westerfield, 2000). D. rerio prrx1 (696 bp), gdf11 (635 bp), hoxc10a (725 bp), sp8 (826 bp) and O. niloticus hoxc10a (718 bp) were amplified by PCR using the following primers, which hybridized to the indicated published sequence: Drprrx1 (ENSAROG0000033971), 5′-GGATGTTAAAAACAAATACTG-3′ and 5′-GACAGTTAGTCCATTGGGTCCT-3′; Drgdf11 (GenBank accession number NM212975), 5′-GCTTATGATCAGTGGAGCA-3′ and 5′-GATACTCTACGCCGCTTATG-3′; Dhoxc10a (GenBank accession number AY591904), 5′-GTGTAGCTGCGCAACTGTC-3′ and 5′-ATACATTTACACATCCAG-3′; Dgdf11 (GenBank accession number AY757342), 5′-GCTTCCGATATCACTGCC-3′ and 5′-AATGATCTTATAACTCCCG-3′. We identified fragments of Ongdf11 using the following primers: outer primer set: 5′-CGNTACCTVAAARATGACCT-3′ and 5′-CCACACGGCCTACCACT-3′; nester primer set: 5′-CGNTACCTVAAARATGACCT-3′ and 5′-TACTCAGTGCACACAGCA-3′. The nucleotide sequences of the Ongdf11 cDNAs were deposited in the GenBank database under the accession number GQ370763.

Microinjection

Morpholino antisense oligonucleotides (MOs) were obtained from Gene Tools, LLC. The following gdf11 MO targeted the boundary between exon 1 and intron 1: MO-gdf11, 5′-CTTGAGCTTTCTAATCTCAGCTCTCT-3′. The standard control MO was used as an injection control. MOs were dissolved in sterile H2O at final concentrations of 4, 5, or 10 mg/ml. Approximately 1 nl of MO was injected at the one-cell stage using a microinjector (IM30, Narishige).

To test the efficiency of gdf11 knockdown by the gdf11 MO, RT-PCR was performed using total RNA from 24 hpf embryos to detect spliced and unspliced gdf11 mRNAs. The following PCR primers were used for amplification: 5′-CATACGAGCGCTATGATCTAC-3′ (exon1), 5′-AGCAAAGACACTTTGTG-3′ (intron1–2), and 5′-CATGGGACCTTACATCATAG-3′ (exon 2). Amplification of eif4α transcript was used as a control (Thisie et al., 2004).

Whole-mount immunostaining

Whole-mount immunostaining was performed as previously described (Crawford et al., 2003), with minor modifications. Larvae for anti-paxillin staining were fixed in 4% paraformaldehyde (PFA) for 3 h at room temperature and then overnight at 4 °C, after which the skin was stripped and washed in phosphate-buffered saline containing 0.05% Tween 20 (PBT). Embryos for anti-i-β-catenin staining were fixed in 4% PFA for overnight at 4 °C and then placed in 0.5 ml of dimethyl sulfoxide (DMSO)/PBT (1:1) on ice until they sank. Then 125 μl of 10% Triton X-100 (Sigma) in distilled water was added, and the embryos were incubated for an additional 20 min at room temperature and then washed in PBT. After blocking with 5% bovine serum albumin (BSA) in PBT, the samples were incubated with the primary antibody (mouse anti-paxillin, diluted 1:200 in 5% BSA in PBT; BD Transduction Laboratory, or rabbit anti-i-β-catenin, diluted 1:200 in 5% BSA in PBT; Sigma) at 4 °C overnight. After washing with PBT, samples were incubated with the secondary antibody (Alexa Fluor 488 goat anti-mouse IgG, diluted 1:250 in 5% BSA in PBT; Invitrogen Molecular Probes, or Alexa Fluor 568 goat anti-rabbit IgG, diluted 1:1000 in 5% BSA in PBT; Invitrogen Molecular Probes) for 2 h at room temperature, washed with PBT, mounted in Vectashield (Vector Laboratories), and observed under a laser confocal microscope (LSM510 META, Carl Zeiss; TCS-SPE, Leica).
Retrograde labeling of neurons

Retrograde labeling experiments were performed essentially as described (Murakami et al., 2004). Biotin-conjugated dextran (Invitrogen Molecular Probes) were injected into the proximal region of the pelvic fins to label motoneurons as described (Glover, 1995) (see also Fig. 6A). The injected embryos were incubated at 28 °C for 10 h to allow the dextran to retrogradely label neurons. Embryos were washed with fish water (Westerfield, 2000), and fixed in 4% paraformaldehyde in PBS at 4 °C overnight. Specimens were then stained as described (Murakami et al., 2001).

Bead transplantation

Acrylic beads (Sigma) were washed three times in PBS and soaked for 20 min in 0.5 mg/ml heparin in PBS at room temperature to enable binding to FGF (Sawada et al., 2001). Heparin-coated acrylic beads were incubated for 2 h in 0.5 mg/ml human recombinant FGF10 protein (R&D Systems) or in PBS at room temperature. Transplantation was performed using a glass needle into the prox1-positive or -negative region of dechorionated embryos at 16 hpf.

Results

Allometric growth of the trunk leads to the rostral shift of presumptive pelvic fin cells

Pelvic fins of most teleosts arise during the larval to juvenile metamorphosis. Observable morphological changes associated with metamorphosis include changes in the resorption of the median caudal fin fold to form the dorsal, caudal, and anal fins, and subsequent formation of soft rays. Interpretation of the evidence regarding the mechanism of the rostral shift of the pelvic fins from a posterior position near the anus in teleost fishes requires examination of the exact position at which pelvic fin buds appear during larval development.

To study the mechanism of the rostral shift of the pelvic fins in teleost development, we compared development of Cypriniformes, zebrafish (Danio rerio), which have abdominal pelvic fins as adults, and Perciforms, Nile tilapia (Oreochromis niloticus), which have thoracic pelvic fins as adults. In zebrafish larvae, signs of metamorphosis from the larval to juvenile transition were observed at ~3 wpf (7.0 mm TL). The formation of soft rays in the proximal part of the resorbed dorsal and anal fins were observed in TL 8.1-mm larvae, whereas no signs of pelvic fins were seen at this stage (Figs. S1A and B). The zebrafish pelvic fin buds were observed at the level of the 8th–9th segments (abdominal level) in TL 8.8-mm larvae (Figs. S1C–F), where pelvic fins were observed in the adult (Figs. S1G and H). In Nile tilapia larvae, pelvic fins appeared on the surface of the yolk just caudal to the pectoral fins at the level of the 3rd–4th segments (thoracic level) at 9 dpf (Figs. S1I–N), as seen in the adult (Figs. S1O and P). The larval pelvic fin buds appeared in the prospective position of the pelvic fins (Fig. S1), as reported (Grandel and Schulte-Mmerker, 1998; Fujimura and Okada, 2007).

We then investigated the distribution of cell populations that participate in pelvic fin formation. First, we examined the fate of lateral plate mesodermal cells in zebrafish embryos by Dil-labeling (Fig. 1). When we labeled the lateral plate mesodermal cells adjacent to somite 14 with Dil (red) at 16 hpf (Fig. 1A), Dil-labeled lateral plate mesodermal cells expanded toward somite 12 at 18 hpf and 25 hpf (Figs. 1B and C) and then reached the somite 9 level at 30 hpf and 36 hpf (Figs. 1D and E). When we examined the fate of cells labeled with Dil opposite somite 14 until 5 dpf in another embryo (Figs. 1F–H), labeled cells expanded toward somite 8 at 30 hpf (Fig. 1G) and toward somite 7 at 5 dpf (Fig. 1H). These results suggest that future pelvic cells reside at the somite 13–14 levels at 16 hpf. We then labeled lateral plate mesodermal cells from the somite 8–9 levels of 16 hpf zebrafish embryos (Fig. 11) and observed their fate. By 30 hpf, labeled cells expanded toward somite 6 (Fig. 1J) and then remained in the same region (Fig. 1K). When we labeled cells at the somite 2 level at 16 hpf, however, labeled cells remained near the labeled area (n=6, Figs. S2A and B). Similarly, we labeled lateral plate cells of 16 hpf zebrafish embryos at different somite levels and observed their fate at various times (Fig. 1P). Dil-labeled cells located caudal to somite 5 seem to have expanded in the mesoderm rostral to the labeled area, and cells from the caudal area tended to have increased rostral expansion compared to those from the rostral area (n=41, Fig. 1P). Notably, the position of most of the cells appeared to have changed by around 30 hpf and remained in the same position at 36 hpf, 3 dpf, 4 dpf and 5 dpf (Fig. 1P). The cells from the somite 13–14 levels reached the prospective pelvic fin position without exception (sample numbers 2–24 in Fig. 1P).

To confirm that the cells adjacent to somite 14 contribute to pelvic fin formation, we injected fluorescent Qtracker reagents (Invitrogen Molecular Probes), an endosome-incorporated long-term tracer (Fig. S2C), into the lateral plate mesoderm next to somite 14 (Fig. 1L). The labeled cells reached the prospective pelvic fin position by 36 hpf (n=15, Fig. 1M). At 1 month post-fertilization (mpf), the labeled cells were observed in the pelvic fins (n=6, Fig. 1N). Thus, in zebrafish, the prospective pelvic fin cells lie next to the anterior border of hox10a expression at 16 hpf. During zebrafish embryogenesis, the yolk tube protrudes from the yolk and extends from 16 hpf; this yolk tube extension ceases at around 30 hpf (Kimmel et al., 1995). Our results raise the possibility that the position of the lateral plate mesodermal cells on the yolk surface shifts with respect to the body trunk concomitant with the trunk–tail protrusion from the yolk (Fig. 1O); such allometric growth of the trunk with respect to the lateral plate mesoderm might lead to the rostral shift of the pelvic fin position among teleost fishes.

In chick and mouse embryos, the positions of the limb buds correlate with the axial level of the spinal cord (Gruss and Kessel, 1991). For example, hindlimb buds of mouse and chick embryos arise in the body wall at the levels of Hox10 paralog expression in the spinal cord (Lance-Jones et al., 2001; Choe et al., 2006). The Dil experiments revealed that the population of cells residing near somite 14 reached the prospective pelvic position. To determine whether prospective pelvic fin cells are at the level of the anterior border of hox10a expression prior to the tail–trunk protrusion, we examined the expression of hox10a during zebrafish embryogenesis (Figs. S3A–D). Interestingly, the anterior limit of hox10a expression in the spinal cord was at the level of somite 14 in 16 hpf zebrafish embryos (Figs. S3A–D). Thus, the presumptive pelvic fin cells may originally have been located at the body wall near the anterior limit of hox10a expression in the spinal cord, as in mouse and chick embryos, prior to the tail bud protrusion.

To investigate whether the presumptive pelvic fins were originally located near the anterior border of hox10 expression despite the difference in the position of the pelvic fins, we examined the distribution of cells that contribute to pelvic fin formation in Nile tilapia embryos, which have thoracic pelvic fins. Because pelvic fin buds appear within the thick “unidentified white tissue” layer on the yolk (Fujimura and Okada, 2007), this thick white tissue should be the lateral plate mesoderm. Thus, we labeled the medial region of the white tissue that was lateral to the body trunk. When Dil was injected into the lateral plate mesodermal cells adjacent to somites 13–14 at 44 hpf (Fig. 2A), labeled cells formed streams of cells in the lateral side of the body and contributed to the pelvic fin by 9 dpf (Figs. 2B and F). Similarly, the Dil-labeled cells opposite somites 13–14 at 44 hpf were found at the somite 10 level by 52 hpf (sample number 5 in Fig. 2F). When we labeled the lateral plate mesodermal cells opposite somite 9 at 52 hpf (Fig. 2C) and examined them at 9 dpf (Fig. 2D) and 12 dpf (Fig. 2F), the labeled cells formed streams running rostrocaudally between the injected region and the pelvic fin buds and contributed to the pelvic fin formation (Figs. 2D and F). When cells from the somite...
Fig. 1. Positional relationships between the body trunk and the lateral plate mesoderm on the yolk change when the trunk–tail protrudes from the yolk during zebrafish development. (A–N) Cell lineage analysis in zebrafish embryos. Locations of Dil-labeled cells (A–K) and quantum dot-labeled cells (L–N) are indicated by arrowheads. (A, F, L) Schematic diagrams showing the locations of Dil or quantum dot (red) application. (A′, F′, L′) Dil or quantum dot was applied to the lateral plate mesoderm cells opposite somites 8–9 (A′) and 14 (F′). (I′) Higher magnification of the labeled regions shown in (F′). (I′) Somatic expression of hoxc10a in the spinal cord at 16 hpf (purple arrowheads), were found at the level of somites 8–9 by 36 hpf and persisted in the same region at 5 dpf, where pelvic fin buds appear (blue arrowheads). (P) Cell lineage analysis in zebrafish embryos. Numbers above each column indicate the opposite somite levels. Expression of hoxc10a in the spinal cord at 16 hpf is indicated by the purple bar. Sample numbers are shown at left. Bold red bars indicate the width of the area labeled with Dil at 16 hpf in each experiment. Light blue, light green, blue, orange, green, grey, yellow and pink bars indicate the width of the area labeled with Dil at 18, 20, 25, 30, 36 hpf, 3 dpf, 4 dpf and 5 dpf, respectively. Note that the Dil-labeled cells around the anterior border of hoxc10a expression in the spinal cord of embryos at 16 hpf were identified in the prospective pelvic fin position by 30 hpf, and continued to be in the same position at 36 hpf, 3 dpf and 5 dpf. Small numbers of lateral plate cells close to the body trunk shift caudally, as they extend together with the body trunk. Scale bars: 100 μm.
9–10 levels were labeled at 51–52 hpf, the cells formed streams and nearly reached the pelvic fin region (sample numbers 7–14 in Fig. 2F). Similarly, we labeled lateral plate cells of 44–48 hpf and 51–52 hpf Nile tilapia embryos at different somite levels and followed their fates (Fig. 2F). Labeled cells expanded rostral to the labeled area in all cases, and cells from the caudal area expanded rostrally more so than did cells from the rostral area (n = 16, Fig. 2F). The position of most of the cells continued to change until at least 9 dpf (Fig. 2F). We also found that the anterior borders of *hoxc10a* expression in the spinal cord of Nile tilapia embryos ranged from the somite 10 to 14 levels, prior to the trunk–tail protrusion (~44 hpf; Figs. S3E–H). Dil-labeling experiments indicated that the lateral plate mesodermal cells located opposite somites 13–14 from 44 hpf, near the anterior border of *hoxc10a* expression, reached to somite 10 by 51–52 hpf, and then likely contributed to the formation of the pelvic fins (Fig. 2E). These results demonstrate that the presumptive pelvic fin cells of teleosts were originally located near the anterior limit of *hoxc10a* expression in the spinal cord despite the difference in the position of pelvic fins—as in tetrapods (Fig. 3). We assume that the presumptive pelvic fin cells, located near the anterior border of *hoxc10a* expression,
receive their positional cue from the body trunk prior to the trunk–tail protrusion.

**Differences in pelvic fin position between zebrafish and Nile tilapia are not due to changes in expression or function of gdf11**

We then tested whether differences in gdf11 expression and/or function could explain the observed differences in pelvic fin position between zebrafish and Nile tilapia. Presumptive pelvic fin cells of early tail-bud stage embryos are originally located at the level of the anterior border of hoxc10a expression in the spinal cord, as in tetrapods. Previous studies in chick and mouse embryos showed that gdf11 expression in the posterior end of the embryos is involved in positioning fore- and hindlimbs via regulation of hox expression (McPherron et al., 1999; Liu, 2006). To test whether pelvic fin position also is controlled by gdf11, we first examined the gdf11 expression pattern in developing zebrafish and Nile tilapia embryos. During embryogenesis of both fish, gdf11 transcripts were located in the caudal end of the embryos (Figs. 4A–F), as seen in chick and mouse embryos (Nakashima et al., 1999; Liu et al., 2001). There were no obvious differences in the expression pattern of gdf11 between zebrafish and Nile tilapia, suggesting that gdf11 expression is not responsible for the observed differences in pelvic fin position between teleosts in a natural system.

We then examined whether the function of gdf11 for fin positioning in zebrafish is common with tetrapods. For this purpose, we blocked gdf11 pre-mRNA splicing (leading to gene product deficiency) during zebrafish development using an antisense morpholino oligonucleotide (MO) to see whether this led to a change in pelvic fin position. Unspliced gdf11 transcripts were detected using RT-PCR in embryos injected with 6 ng of the gdf11 MO, whereas both unspliced and spliced gdf11 mRNAs were detected in embryos injected with control MO (Fig. 5A), demonstrating that the gdf11 MO efficiently blocked gdf11 mRNA maturation. We then examined the effect of gdf11 on hoxc10a expression in zebrafish embryos after injection of 4 ng of gdf11 MO. At 16 hpf, the caudal displacement in the expression of hoxc10a transcripts was observed in 55 of 62 embryos (Figs. 5B and C). Measurement of the caudal shift of hoxc10a expression revealed that the injection of 10 ng or 4 ng of gdf11 MO leads to the equivalent of 1–2 segments of caudal displacement of hoxc10a expression (Fig. 5A). If the fate of presumptive pelvic fin cells is not established during early development, at least until 1 dpf, injection of gdf11 MO could lead to a caudal shift in pelvic fin position. To explore this, we incubated zebrafish embryos injected with 4 or 10 ng of gdf11 MO for 44–60 days until the pelvic fins were formed. In control morphants, the pelvic fins were seen in the abdominal region at the 8–9 segment levels (n=40, Fig. 5D, Figs. S5A and C), whereas pelvic fins were seen at the 9–10 segment levels in 78.5% (51/65) of embryos injected with 10 ng of gdf11 MO (Fig. 5E, Figs. S5B and C). Similarly, a caudal shift in pelvic fin positions was also observed in 54.5% (30/55) of embryos injected with 4 ng of gdf11 MO (Fig. 5F).

We then labeled the lateral plate mesodermal cells of embryos injected with 10 ng gdf11 MO to investigate whether cells at the anterior margin of hoxc10a expression reached the prospective pelvic fin position (n=8, Figs. S4B–E). Dil-labeling experiments indicated that the lateral plate mesodermal cells located opposite somite 15, near the anterior margin of the border of hoxc10a expression in gdf11 morphants, reached the 9–10 somite levels, where pelvic fins appear in gdf11 morphants (Figs. S4B–E). These results demonstrate that reducing gdf11 expression in zebrafish embryos can induce caudal displacement in hoxc10a expression domains and cause a caudal shift in pelvic fin position (Fig. 5F). Thus, in teleost fishes, the mechanism that positions the paired appendages via modulation of gdf11 expression levels seems to be common with tetrapods. Based on these findings we conclude that differences in pelvic fin position between teleosts are not due to changes in gdf11 expression or function, but rather some other unknown mechanism.

**Motoneurons lying above the pelvic fins innervate the pelvic muscles**

In chick and mouse embryos, Hox10 expression corresponds to the position of the LMC motoneurons that innervate the limb musculature (Lance-Jones et al., 2001; Shah et al., 2004; Choe et al., 2006). To investigate whether the motoneurons that innervate the musculature of pelvic fins reside at the anterior border of hoxc10a expression in teleost fishes as in tetrapods, retrograde labeling of motoneurons with
A common mechanism underlies the positioning of paired appendages via gdf11 expression levels in both teleosts and tetrapods. (A) RT-PCR analysis to determine the efficiency of the gdf11 splice-blocking morpholino (MO). In the schematic diagrams, arrows represent forward and reverse primers, and the short red bar represents the gdf11 MO. RT-PCR products were analyzed by agarose gel electrophoresis. Products of 392 bp and 525 bp represent spliced and unspliced gdf11 mRNA, respectively. Amplification of eif4a cDNA was used as a control.

(B, C) Expression of hoxc10a in 16 hpf embryos injected with control MO (B) and gdf11 MO (C). The orange dashed line indicates the hoxc10a positive region of control morphant in (B). Caudal displacement of the anterior border of hoxc10a expression in the spinal cord (white arrowheads) is seen in the gdf11 morphant (C).

(D, E) Scanning micrographs of zebrafish larvae. (D) Control (TL 10.5 mm). (E) gdf11 morphant (TL 10.1 mm). (F) Diagrams show the domains of hoxc10a expression in the spinal cord (purple) of control and gdf11 morphants. The anterior border of hoxc10a expression in the spinal cord of the control (opposite somite 14) and of gdf11 morphants are indicated by purple and red arrowheads, respectively. Pelvic fins of control larvae appear opposite the 8th-9th segments (blue); pel, pelvic fin. Scale bars: 100 μm for panels (B) and (C); 500 μm for panels (D), (E) and (F).

Fig. 4. gdf11 is expressed in the posterior end of the body in both zebrafish and Nile tilapia embryos. (A–C) Expression of gdf11 in zebrafish embryos at the times indicated. (D–F) Expression of gdf11 in Nile tilapia embryos at the times indicated. There was no significant difference in the efficiency of gdf11 transcripts between zebrafish and Nile tilapia embryos. Scale bars: 100 μm for panels (A), (B) and (C); 500 μm for panels (D), (E) and (F).

Fig. 5. A common mechanism underlies the positioning of paired appendages via gdf11 expression levels in both teleosts and tetrapods. (A) RT-PCR analysis to determine the efficiency of the gdf11 splice-blocking morpholino (MO). In the schematic diagrams, arrows represent forward and reverse primers, and the short red bar represents the gdf11 MO. RT-PCR products were analyzed by agarose gel electrophoresis. Products of 392 bp and 525 bp represent spliced and unspliced gdf11 mRNA, respectively. Amplification of eif4a cDNA was used as a control. (B, C) Expression of hoxc10a in 16 hpf embryos injected with control MO (B) and gdf11 MO (C). The orange dashed line indicates the hoxc10a positive region of control morphant in (B). Caudal displacement of the anterior border of hoxc10a expression in the spinal cord (white arrowheads) is seen in the gdf11 morphant (C).

(D, E) Scanning micrographs of zebrafish larvae. (D) Control (TL 10.5 mm). (E) gdf11 morphant (TL 10.1 mm). (F) Diagrams show the domains of hoxc10a expression in the spinal cord (purple) of control and gdf11 morphants. The anterior border of hoxc10a expression in the spinal cord of the control (opposite somite 14) and of gdf11 morphants are indicated by purple and red arrowheads, respectively. Pelvic fins of control larvae appear opposite the 8th-9th segments (blue); pel, pelvic fin. Scale bars: 100 μm for panels (B) and (C); 500 μm for panels (D), (E) and (F).
somites 3–4 innervated the pelvic fins (n = 9; Figs. 6G–K). We then observed expression of hoxc10a in Nile tilapia embryos. At 42 hpf, the anterior border of hoxc10a in the spinal cord reached the most rostral level, somite 10, but never reached somite 3 and 4 levels during embryogenesis (Figs. S3E–H). Thus, the anterior margin of hoxc10a expression in the spinal cord of zebrafish and Nile tilapia did not correspond to the prospective pelvic fin position at any stage. These results suggest that, in teleost fishes, unlike tetrapods, the motoneurons residing above the pelvic fins, but not at the anterior border of hoxc10a expression, innervate the pelvic muscles. In other words, the hox-dependent mechanism by which teleost fishes specify the motoneuron along the rostrocaudal axis has not been established with regard to pelvic fin innervation.

Discussion

Our study yielded the following findings. (1) Presumptive pelvic fin cells reside adjacent to the anterior border of hoxc10a expression in the spinal cord, and their position shifts with respect to the trunk. (2) Differences in pelvic fin position between zebrafish and Nile tilapia were not due to changes in expression or function of gdf11. (3) The hox-dependent mechanism that specifies the motoneuron along the rostrocaudal axis is not established for teleost pelvic fin innervation.

Rostral shift of the pelvic fins in teleost fishes

The pelvic fins of some teleost fishes have shifted rostrally during evolution. The resultant diversification of pelvic fin position among teleosts allowed them to occupy diverse habitats (Rosen, 1982). Here we showed that presumptive pelvic fin cells resided next to the anterior border of hoxc10a expression, as in tetrapods, but the positional relationship between the presumptive pelvic fin cells on the yolk surface and the body trunk was altered during development, causing the rostral shift of the pelvic fins.

In tetrapods, the posterior paired appendages are usually located next to the anus, whereas in teleost fishes, the pelvic fins arise in front of the anus. This rostral shift of pelvic fin position in teleost fishes seems to have occurred because the teleost lateral plate mesodermal cells on the yolk surface cannot grow with the body trunk at the same rate as observed in tetrapods. Alternatively, it may have been a consequence of the fact that the lateral plate mesodermal layer of teleosts is not divided into somatic and splanchnic layers at the time of trunk protrusion, so that the undivided lateral plate layer attached to the yolk surface cannot grow with the body trunk. In zebrafish embryos, there are only a few layers of lateral plate mesodermal cells on the yolk surface (Grandel and Schulte-Merker, 1998; Fig. S6). The distribution of prpx1, the lateral plate mesodermal marker, showed that the population of prpx1-positive cells narrows toward the caudal region when the body trunk grows (Fig. S6). This seems to be caused by the allometric growth of the body trunk in relation to the lateral plate mesoderm (Fig. 1), which was also observed during Nile tilapia development (Fig. 2). Thus, our results suggest that the allometric outgrowth of the body trunk with respect to the lateral plate mesoderm can lead to the rostral shift of pelvic fins relative to the anus in teleost fishes.

Our conclusions provide fundamental insights into the mechanisms of diversification of pelvic fin positions. We now have to consider...
the allometric growth of the trunk as one of the potential reasons why pelvic fin position varies among fish species. Differences in migration speed and/or the path of lateral plate mesodermal cells over the yolk could also be critical for the diversification of pelvic fin positions. Although it is unlikely, we cannot exclude the possibility that the differences in yolk shape are critical for the rostral shift of pelvic fins. The yolk of zebrafish is undercut at the time of the trunk–tail protrusion and forms a yolk tube, whereas Nile tilapia forms a large spheroidal yolk and does not form a yolk tube. Further embryological and molecular studies in various teleost species are required to determine what governs the difference in the position of pelvic fins.

Our results show that the allometric growth of the trunk causes the rostral shift of the lateral plate mesoderm with respect to the trunk. This also raises interesting questions about the distribution of the ectoderm overlying the lateral plate mesoderm. It has been shown that the lateral plate mesoderm along the body side has widespread limb-forming potential in various tetrapods, including chick and mouse embryos (Cohn et al., 1995; Ohuchi et al., 1995; Tanaka et al., 2000). In chick and mouse embryos, the region of the body where an apical ectodermal ridge of an ectopic limb bud can be induced corresponds precisely to the region where Engrailed-1 is expressed in the ventral side of the ectoderm overlying the lateral plate mesoderm (Davis et al., 1991; Loomis et al., 1996). Such expression patterns of Engrailed genes in the ventral half of the body ectoderm have been observed in cartilaginous dogfish embryos (Tanaka et al., 2002) and on the ventral side of the body of agnathan lamprey embryos (Matsuura et al., 2008). On the other hand, in teleost fish body ectoderm the expression of engrailed has not been reported despite the expression of Engrailed-1a in the ventral ectoderm of the pectoral fin bud in zebrafish embryos (Ekker et al., 1992). Interestingly, when we implanted FGF-soaked beads in the prrx1-positive region of zebrafish embryos, ectopic sp8 (the apical ridge marker) expression was observed only on the line extending from the ridge of pectoral fin buds, regardless of the site of the implanted beads (Fig. S7), suggesting that ectopic apical ridges might have been induced only in the dorsoventral boundary of the overlying ectoderm in zebrafish. If the ectoderm overlying the lateral plate mesoderm (prrx1-positive region) shifted the apical ridge position relative to the underlying mesoderm during trunk protrusion, the dorsoventral boundary of the ectoderm should have been observed on the line extending from the pectoral fins to the pelvic fins. It would be interesting to know whether the body ectoderm of embryos and larvae of teleosts with various pelvic fin positions are compartmentalized.

**Positioning of paired appendages in vertebrates**

We observed the caudal displacement of pelvic fin position in zebrafish embryos injected with gdf11 MO (Fig. 5). This change in fin position was accompanied by the caudal shift of hoxc10a expression in the spinal cord and paraxial mesoderm. In mice and chick embryos, altered gdf11 expression displaces axial Hox expression and limb position. The limbs of mice lacking Hoxb5 are also shifted rostrally. Our results suggest that, to some extent, there is a mechanism common to both teleosts and tetrapods that controls the positioning of paired appendages via modulation of gdf11 expression. The mechanism that determines limb position in tetrapods is not fully understood. Displacement of limbs in chick, mice and zebrafish embryos by modulating gdf11 expression could be caused by changes in axial Hox expression, by an effect of Gdf11 secreted from the spinal cord and/or paraxial mesoderm in the lateral plate mesoderm, or by changes in the levels or patterns of unknown factors controlled by Gdf11 or Hox.

Our results suggest that the presumptive pelvic fin cells originally reside next to the anterior border of hoxc10a expression, as in tetrapods, prior to the trunk–tail protrusion. Furthermore, we show that there is a common mechanism involving gdf11 that controls pelvic fin position in both teleosts and tetrapods. Thus, we assume that the presumptive pelvic fin cells receive the same type of positional cue, as tetrapods, from the body trunk when they reside next to the anterior border of hoxc10a in the spinal cord. This cue is probably a factor(s) controlled by gdf11 or gdf11 itself secreted from the body trunk. However, we cannot rule out the possibility that such a positional cue is provided after the trunk–tail protrusion, because 4 ng of gdf11 MO blocked splicing until at least 60 hpf (data not shown). We also cannot exclude the possibility that cells also receive an undetermined positional cue at the time of pelvic fin initiation. In future work, it will be important to uncover the mechanism by which the presumptive limb/fn cells receive the positional cue from the body trunk, which could reveal the relationships between hox expression in the spinal cord, gdf11 expression in the spinal cord and the paraxial mesoderm, and the genes associated with limb/fn initiation in the lateral plate mesoderm.

**Evolution of the motoneurons**

Our data demonstrate that the motoneurons residing directly above the pelvic fins, but not those at the hoxc10a expression boundary at any stage, innervate the pelvic musculature in zebrafish and Nile tilapia larvae. In tetrapods, Hox10 paralogs specify the LMC at the lumbar level in the spinal cord, and the motoneurons in the lumbar–lateral motor column innervate the musculature of hindlimbs. Such a Hox-dependent rostral–caudal specification of motoneuron columns has been suggested to have evolved in parallel with the emergence of novel target structures. Primitive aquatic vertebrates, including teleost fishes and lampreys, seem to lack LMCs but have Median motor column (MMC) and Hypaxial motor column (HMC) (Fetcho, 1992; Kusakabe and Kuratani, 2005). Emergence of the LMC is associated with the acquisition of paired limbs during vertebrate evolution (Fetcho, 1992). Recently, Dasen and his colleagues showed that the Hox accessory factor, FoxP1, is expressed in PGC and LMC at different levels and specifies those motor columns, but is not expressed in the primitive types of motor columns, such as MMC and HMC. Furthermore, LMC of mice lacking FoxP1 exhibits HMC-like characteristics, and such HMC-like motoneurons generated at limb levels innervate the encountered limb musculature (even though the topology of axonal trajectories may be scrambled) but not the inter-limb body wall (Dasen et al., 2008). These facts are compatible with our current findings that motoneurons of zebrafish and Nile tilapia, which lack the LMC, innervate the musculature of pelvic fins in a manner independent of the rostral–caudal patterns of hox expression in the spinal cord. Further molecular and developmental approaches to the evolution of motor column specificity and connectivity in relation to limb evolution should provide clues to the process of paired appendage evolution in vertebrates.

We have shown that prospective pelvic fin cells on the yolk surface shift their positional relationships with respect to the body trunk because of the allometric growth of the trunk. Such allometric growth is thought to underlie evolutionary morphological innovations in the internal cheek pouch of kangaroo rats and hamsters (Bryliski and Hall, 1988) and the nose of whales on the top of the skull (Slijper, 1962). Our study suggests that the allometric growth of the trunk may also be involved in the major morphological changes in the pelvic fin position during evolution of teleost fishes.

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References


