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Developmental genetic basis for the evolution of pelvic fin loss in the pufferfish *Takifugu rubripes*

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Abstract

Paired appendages were a key developmental innovation among vertebrates and they eventually evolved into limbs. Ancient developmental control systems for paired fins and limbs are broadly conserved among gnathostome vertebrates. Some lineages including whales, some salamanders, snakes, and many ray-fin fish, independently lost the pectoral, pelvic, or both appendages over evolutionary time. When different taxa independently evolve similar developmental morphologies, do they use the same molecular genetic mechanisms? To determine the developmental genetic basis for the evolution of pelvis loss in the pufferfish *Takifugu rubripes* (fugu), we isolated fugu orthologs of genes thought to be essential for limb development in tetrapods, including limb positioning (*Hoxc6*, *Hoxd9*), limb bud initiation (*Pitx1*, *Tbx4*, *Tbx5*), and limb bud outgrowth (*Shh*, *Fgf10*), and studied their expression patterns during fugu development. Results showed that bud outgrowth and initiation fail to occur in fugu, and that pelvis loss is associated with altered expression of *Hoxd9a*, which we show to be a marker for pelvic fin position in three-spine stickleback *Gasterosteus aculeatus.* These results rule out changes in appendage outgrowth and initiation genes as the earliest developmental defect in pufferfish pelvic fin loss and suggest that altered *Hoxd9a* expression in the lateral mesoderm may account for pelvis loss in fugu. This mechanism appears to be different from the mechanism for pelvic loss in stickleback, showing that different taxa can evolve similar phenotypes by different mechanisms.

Keywords: *Hox* genes; Limb positioning; Limb initiation; Fugu; *Takifugu rubripes*; *Gasterosteus aculeatus*; Stickleback; Fin development; Evolution of development

Introduction

The evolution of paired pectoral and pelvic fins improved the ability of ancient vertebrates to maneuver, making them mobile, fearsome predators. Paired fins evolved into limbs as vertebrates adapted to terrestrial environments, losing fin rays, and gaining digits. The ancestral developmental genetic system leading to appendage bud positioning, initiation, and outgrowth was widely retained among gnathostome vertebrates (Akimenko and Ekker, 1995; Hinchliffe, 2002; Sordino et al., 1995). Subsequently, one or both paired appendages were secondarily lost in many vertebrate lineages, including snakes, salamanders (e.g., Sirenidae), lizards (e.g., Amphisbaenidae), mammals (e.g., whales), and independently in several lineages of ray-fin fish, including pufferfish, cowfish, and some stickleback populations (Bell et al., 1993; Santini and Tyler, 2003). What is the molecular genetic basis for the evolution of appendage loss? Is appendage loss in different lineages due to independent evolution of the same developmental genetic mechanism? Or did different lineages lose paired appendages by evolving

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changes in genes affecting different levels of the broadly conserved genetic regulatory system for appendage development? To answer these questions, we compared molecular aspects of pelvic fin development in two teleosts, stickleback and pufferfish, both members of the Series Percomorpha (Nelson, 1994).

Embryonic development of vertebrate paired appendages passes through three main phases—positioning, initiation, and outgrowth (Fig. 1). We designed experiments to test the hypotheses that the evolution of pelvic reduction in the pufferfish lineage occurred due to the selection of regulatory mutations altering the positioning, or the initiation, or the outgrowth of the appendage bud. In the positioning phase, paired appendages arise from bud initials that form in the lateral plate mesoderm at positions thought to be specified by *Hox* gene expression in somitic mesoderm (Burke et al., 1995; Rancourt et al., 1995). In tetrapods, the position of the forelimb is associated with the anterior expression border of *Hoxc6* in the paraxial mesoderm (Burke et al., 1995), and the position of the forelimb, interlimb region, and hindlimb is associated with expression of *Hoxb9*, *Hoxc9*, and *Hox9d* in the lateral plate mesoderm (Cohn et al., 1997).

The initiation phase of limb development follows the positioning phase (see for review (Tanaka and Tickle, in press)). The transcription factor genes *Tbx5* and *Tbx4* are expressed in forelimb and hindlimb buds, respectively, at the time of limb field specification in tetrapods and zebrafish (Chapman et al., 1996; Tamura et al., 1999), where they specify limb identity (Logan and Tabin, 1999; Rodriguez-Esteban et al., 1999). *Tbx4* expression in the hindlimb is regulated by *Pitx1* and to a lesser extent *Pitx2* (Lanctot et al., 1999; Logan and Tabin, 1999; Marcil et al., 2003).

In developing forelimbs, the role of *Tbx5* in appendage initiation and outgrowth is mediated by interactions with *Wnt2b* and *Fgf10* (Agarwal et al., 2003; Takeuchi et al., 2003), while in chick embryonic hindlimbs, *Tbx4* lies upstream of *Wnt8c* and *Fgf10* (Takeuchi et al., 2003).

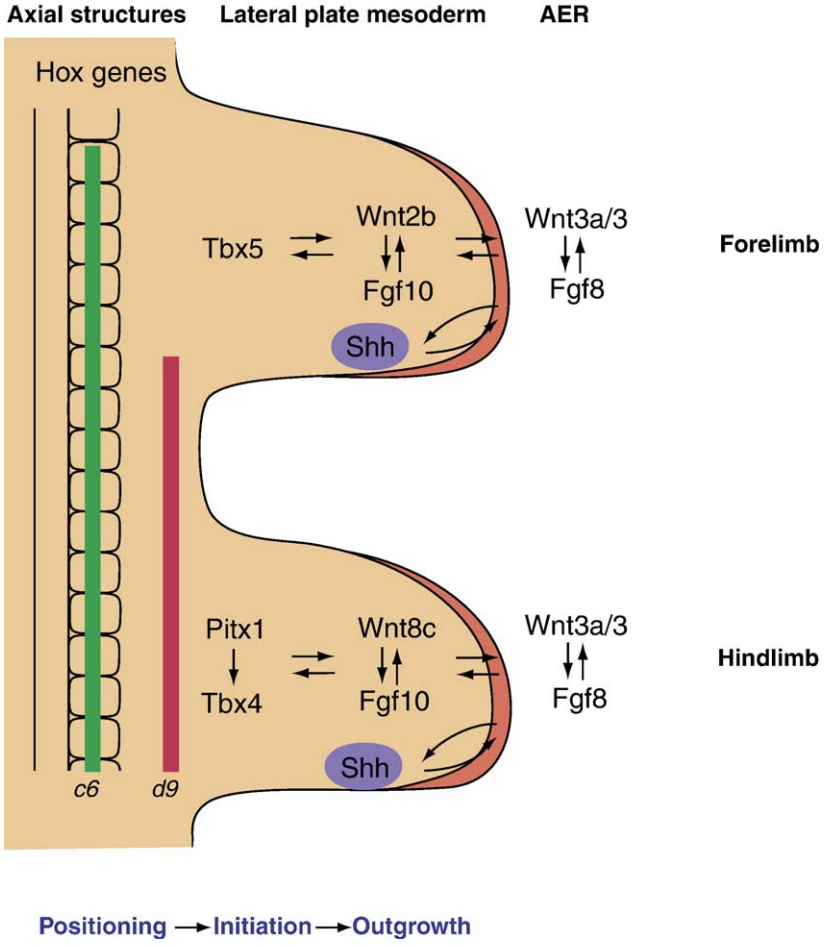

Fig. 1. Key genes in limb development. Hox gene expression in somitic mesoderm and in lateral plate mesoderm specifies position in the head to tail body axis and the locations of the forelimb, interlimb region, and hindlimb. Subsequently, in lateral plate mesoderm, Tbx5 and Tbx4 activate Wnt2b/Fgf10 and Wnt8c/Fgf10 signals in forelimb and hindlimb, respectively. Wnt/Fgf signals feedback on Tbx5 and Tbx4 to maintain their expression. Fgf10 activates Wnt3a/3/Fgf8 signals in the limb ectoderm and induces the formation of apical ectodermal ridge *Shh* expression in the posterior limb bud is maintained by FGF signaling by the apical ectodermal ridge, and FGF signals feed back on *Shh* to maintain its expression. Modified from (Tanaka and Tickle, in press).

The initiation phase and the following outgrowth phase of limb development involve fibroblast growth factors (FGFs), which can induce ectopic limb buds in chick and mouse embryos (Cohn et al., 1995; Ohuchi et al., 1995; Tanaka et al., 2000; Yonei-Tamura et al., 1999). *Fgf8* expression in the apical ectodermal ridge (AER) of the limb bud (Fig. 1) reciprocally maintains expression of *Fgf10* and promotes limb outgrowth (Ohuchi et al., 1995). Wnt signaling can also trigger limb development by inducing *Fgfs* in chick embryos (Barrow et al., 2003; Kawakami et al., 2001). FGF signaling from the apical ectodermal ridge is critical for sustaining limb outgrowth. Reciprocal signaling by the mesenchyme and *Shh* expression at the posterior margin of the limb bud maintains FGF signaling at the apical ectodermal ridge (Niswander et al., 1994).

The molecular genetic basis of limb loss has been studied in few lineages. In python snakes, the expression of *Hox* genes appropriate for interlimb flank identity expands rostrally, resulting in the loss of axial positions suitable for pectoral limb initiation (Cohn and Tickle, 1999). Although positioning cues for pectoral limbs are altered in pythons, outgrowth cues are aberrant in python pelvic limb buds, which initiate development, but fail to sustain outgrowth associated with inactivation of outgrowth signaling pathways (Cohn and Tickle, 1999). Like pythons, whale embryos initiate pelvic limb buds that fail to continue development (Bejder and Hall, 2002). Squamate reptiles show various degrees of limb and girdle loss (Greer, 1991). Embryos of serpentiform lizards initiate limb buds and develop an apical ectodermal ridge that then regresses (Raynaud, 1990). In the Australian lizard *Hemiergis*, the duration of *Sonic hedgehog* expression is shortened in limbs with a reduced number of digit (Shapiro et al., 2003). In threespine stickleback, many fresh water populations have evolved pelvic appendage loss from an anadromous ancestor (Bell and Orti, 1994). Gene expression studies showed that *Pitx1*, a gene necessary for appendage initiation, fails to be expressed in stickleback from fresh waters of Scotland with pelvic reduction (Cole et al., 2003). In Alaskan stickleback, a single Mendelian locus of strong effect governs pelvic development (Cresko et al., 2004), and in a Canadian population, a quantitative trait locus with major effect on the pelvic apparatus maps at or near the pelvic reduction gene in the Alaskan population (Shapiro et al., 2004). The major effect locus in stickleback maps at the same end of a chromosome as *Pitx1*, but the *Pitx1* gene encodes a normal protein in pelvic-reduced Canadian populations (Shapiro et al., 2004). It is not yet known if a cis-acting site near the *Pitx1* gene that is important for limb formation is altered, leading to failure of pelvic development in these fresh water populations.

Adult pufferfish *Takifugu rubripes* (fugu) and related fish, including porcupine fish and ocean sunfish (mola) lack a bony pelvic apparatus (Santini and Tyler, 2003). Here, we use molecular markers to test whether pelvic fin loss in pufferfish is due to truncation of the developmental program at the positioning, initiation, or outgrowth phase of fin development. As a positive control, we followed pelvic fin development in anadromous three-spine stickleback *Gasterosteus aculeatus*, which develop a robust pelvic apparatus. Results showed that the positioning of the pelvic apparatus in stickleback is associated with the novel expression of *Hoxd9* in the lateral plate mesoderm in mid-metamorphosis. Pelvic fin loss in *Takifugu rubripes* is associated with the lack of this expression of *Hoxd9* as a marker for pelvic fin position and the subsequent failure to activate signaling pathways for pelvic fin initiation and outgrowth.

Experimental procedures

Pufferfish and stickleback embryos and larvae were reared as described (Cresko et al., 2004; Suzuki et al., 2002). Cartilage staining was conducted according to (Kimmel et al., 1998). Scanning electron microscopy was performed as previously described (Cole et al., 2003). Probes for in situ hybridization experiments were constructed from *T. rubripes* (Aparicio et al., 2002) and *G. aculeatus* genomic DNA (Cresko et al., 2003) and used for in situ hybridization experiments as described (Jowett and Yan, 1996). The fugu genes *TrShh* (SINFRUG00000125602), *TrFgf10* (SINFRUG00000135164), *TrPitx1* (SINFRUG00000134231), *TrTbx5* (SINFRUG00000146715), *TrTbx4* (SINFRUG00000 00000132010), *TrHoxc6* (SINFRUG00000146326), *TrHoxd9a* (SINFRUG00000124776) and *TrHoxd9b* (SINFRUG00000156364) are on scaffolds 3696, 1074, 527, 218, 672, 285, 214 and 19911, respectively. Sequences for primers were *TrShh* (5′-ATCCACTCGGGATGTCAGTATC-3′ and 5′-GTTGCACATGTATGCACATATG-3′), *TrFgf10* (5′-GTCAGGTCTGAATGATGCACTG-3′ and 5′- TGATGGAAGGTTGTAGTATTGG-3′), *TrPitx1* (5′-TTCACGCACCTGAATATGCGAG-3′ and 5′- ACTGA-TGGACTCAGCGTT CGC-3′), *TrTbx5* (5′- TGCTGCTTGTCATCCTAATGAG-3′ and 5′-GAAAGTAAGTTGCTGTAACTAC-3′), *TrTbx4* (5′-CCAGCTCAGTCGAGAC-ACACAG-3′ and 5′-GGCTCATGCGTCCATCCGTCAC-3′), *TrHoxc6* (5′-AACCTGACCTCCACGGTGACTG-3′ and 5′-ATGCAGACAGTTCAAGTAGTAG-3′), *TrHoxd9a* (5′- GCGTCCGCTCCTGGATGGAGCC-3′ and 5′-AATCTCATCAATAAGGAGCAGC-3′), *TrHoxd9b* (5′-ACGAATAAGGTCCTAGC TGTGG-3′ and 5′-GCCGTCGATACAACTACGATTC-3′), *GacHoxc6* (5′-CTGGTTTCAGAACCGACGTAT-G-3′ and 5′-ATGATTCAACTAGGCCTACTAC-3′) and *GacHoxd9a* (5′-GCGGAGCTCAGTGGAAGTGAAC-3′ and 5′-ATACAATGGCTACCGTTGCTCC-3′). The nucleotide sequences of *TrShh, TrFgf10, TrPitx1, TrTbx5, TrTbx4, TrHoxd9a, TrHoxd9b, TrHoxc6, GacHoxc6* and *GacHoxd9a* are deposited in the GenBank database under accession numbers AY829360–AY829367, AY744146 and AY744145.

Results and discussion

Skeletogenesis

Interpreting evidence regarding the molecular mechanism of pufferfish pelvic loss requires examination of stages at which the pelvic apparatus develops in outgroup lineages that have pelvic fins. Anadromous stickleback make a suitable comparator because pufferfish and stickleback occupy two orders within the Series Percomorpha, a teleost taxon characterized by an anterior shift of the pelvic apparatus from a posterior position near the anus, as in zebrafish and most other teleosts, to thoracic or jugular positions (Nelson, 1994). Furthermore, the development of the stickleback pelvis has been thoroughly investigated (Baker et al., 1995; Bell, 1987; Bell et al., 1993; Cole et al., 2003; Cresko et al., 2004; McPhail, 1992; Reimchen, 1997; Shapiro et al., 2004). To standardize life cycles across teleosts, we chose the events of metamorphosis, which occur in roughly the same order in different teleosts. Readily observable morphological changes associated with metamorphosis from larval to juvenile teleosts include changes in the pigment pattern (Johnson et al., 1995; Parichy and Turner, 2003), resorption of the median caudal fin fold to form the dorsal, caudal, and anal fins (Johnson and Weston, 1995), and appearance of the pelvic apparatus (Brown, 1997; Swarup, 1958).

Although the developmental time table of stickleback is longer than that of zebrafish (Swarup, 1958), in both teleosts, the pectoral fin develops before hatching, but the pelvic fin arises during metamorphosis (van Eeden et al., 1996). The stickleback pelvic fin bud is apparent in scanning electron micrographs just caudal to the pectoral fins (arrowhead in Fig. 2b), while the median fin fold is still metamorphosing into dorsal, caudal, and anal fins (Fig. 2a) and the pigment pattern is still metamorphosing, as melanophores invade the flank and arrange in dorsal–ventral stripes. We conclude that in stickleback embryos,

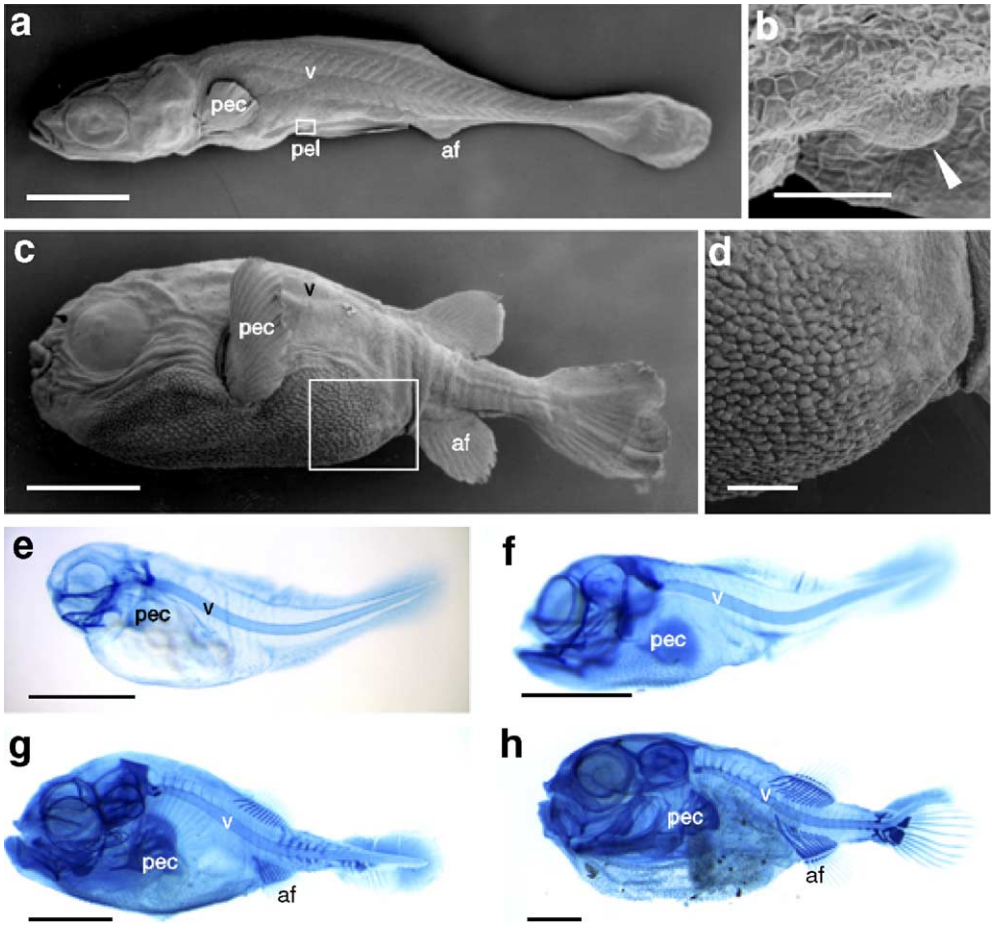

Fig. 2. Scanning electron microscopy and skeletal anatomy of stickleback and fugu. (a–d) Scanning micrographs of late metamorphosis stage stickleback and fugu larvae. (a) Stickleback larva. 6 mm SL (standard length, measured from the tip of the snout to the posterior extremity of the hypurals, the expanded bones at the end of the backbone that support the caudal fin), scale bar 1 mm. (b) Detail of pelvic fin region in (a), showing pelvic fin bud outgrowth (arrowhead) before metamorphosis of medial caudal fin fold. Scale bar 100 μm. (c) Fugu larva. SL 3.6 mm, scale bar 1 mm. (d) Detail of pelvic region in (c), showing no evidence of pelvic fin bud. Scale bar 100 μm. (e–h) Cartilage pattern in Alcian blue stained fugu larvae. (e) 7 days. Scale bar 500 μm, SL 1.8 mm; (f) 14 days, scale bar 500 μm, SL 2.5 mm; (g) 21 days, scale bar 500 μm, SL 2.1 mm; (h) 28 days, scale bar 500 μm, SL 3.6 mm. Fugu larvae did not form cartilaginous elements corresponding to the pelvic region at any stages examined. Abbreviations: pec, pectoral fin; pel, pelvic fin; v, vertebrae; af, anal fin.

outgrowth of the pelvic fin bud occurs about midway during the process of metamorphosis. In our fugu samples, the dorsal half of the young larvae is transparent, and the ventral half is dark due to embryonic melanophores. Between 3 and 4 weeks after fertilization, adult-type melanophores differentiate in the dorsal portion of the animal to produce black spots and embryonic melanophores disappear from the ventral side. During this same time period, the median fin fold resolves into the dorsal, caudal, and anal fins. We examined fugu larvae from hatching until after metamorphosis, and at no stage did they show a pelvic fin bud similar to that we observed in marine stickleback (Figs. 2a–d). We conclude that pelvic development in fugu aborts before the visible outgrowth of a fin bud.

To test the hypothesis that the pelvic apparatus of larval fugu forms internal skeletal elements of the pectoral girdle that fail to become apparent in an external appendage bud, we examined fugu embryos stained with Alcian blue to investigate cartilage patterns at various stages of development. Results showed that, although Alcian staining tracked the developmental profile of the pectoral fin and girdle, it revealed no signs of cartilage formation in the pelvic region at any stage examined (Figs. 2e–h). Because pelvic fin buds appear in anadromous stickleback and zebrafish during metamorphosis, and because we examined pufferfish larvae past this stage, we conclude that pelvic fin loss in fugu is due to a failure of fin bud positioning, bud initiation, or early stages of outgrowth rather than a truncation of limb bud extension or later stages in skeletogenesis.

Bud outgrowth

In tetrapods and ray-fin fish, mesenchymal cells in the zone of polarizing activity at the posterior margin of paired appendages express *Sonic hedgehog* (*Shh*) early in the outgrowth phase, and the encoded extracellular protein specifies pattern along the appendage's anterior–posterior axis (Akimenko and Ekker, 1995; Riddle et al., 1993). The zone of polarizing activity and the apical ectodermal ridge mutually maintain each other, mediated by feedback mechanisms between SHH and FGF4 (Niswander et al., 1994), coordinating limb outgrowth and patterning. To test the hypothesis that this developmental signaling system is defective in fugu, as it is in hindlimb buds of pythons (Cohn and Tickle, 1999), we isolated portions of the *Shh* gene from fugu (Goode et al., 2003; Lettice et al., 2003) and examined *Shh* expression in fugu embryos and larvae. Results showed that five-day old fugu embryos express *Shh* in the floor plate of the neural tube and notochord (Fig. 3a) and in the zone of polarizing activity of the pectoral fin buds (arrows in Fig. 3b), as they do in zebrafish (Krauss et al., 1993). In contrast, no *Shh* transcripts could be detected in the pelvic area in fugu embryos at any stage examined up to 4 weeks (Fig. 3c), which is past the developmental stage in which *Shh* is active in the pelvic fin buds of zebrafish (Sordino et al., 1995). Thus, pelvic fin formation in fugu is disturbed prior to the establishment of the polarizing region.

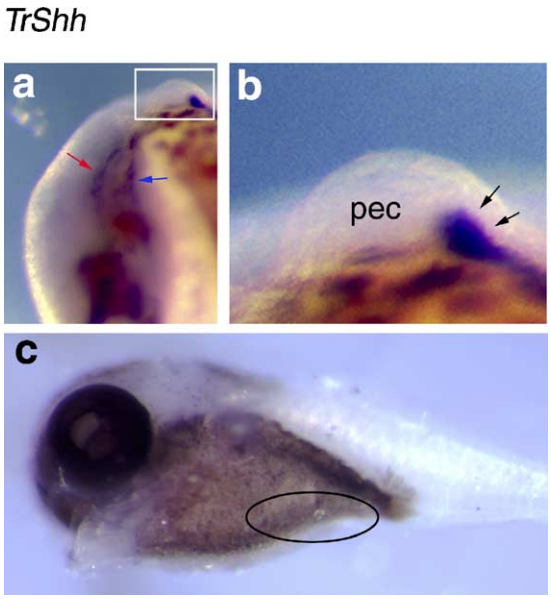

Fig. 3. Expression pattern of *Shh* in fugu. (a–c) Expression of *TrShh*, the *T. rubripes Shh* gene. (a) Lateral view of 5-day-old larva. Red and blue arrows indicate the nervous system and gut, respectively. (b) Detail of pectoral fin bud region in (a). Arrows indicate *TrShh* transcripts in the posterior margin of the fin bud. (c) Ventral view of 14-day-old larva. Note absence of *TrShh* transcripts in the pelvic region (circled).

Bud initiation

Having shown that later stages of pelvic development were disrupted, including skeletogenesis and bud outgrowth, we next investigated whether the pelvic apparatus aborts in stickleback during the bud initiation phase. We isolated the fugu orthologs of genes known to be involved in limb initiation in tetrapods, and examined their expression patterns at various stages (Fig. 4). *FGF10* encodes a diffusible signaling protein. Fugu *Fgf10* (SINFRUG00000135164) is on the end of Scaffold_1074, and its nearest neighbor encoding the transcript SINFRUT00000143233 is orthologous to *NNT*, which is the nearest neighbor to *FGF10* in the human genome at 5p13–p12; this conserved synteny information confirms our orthology assignment. In situ hybridization experiments showed that *Fgf10* was expressed in mesoderm of pectoral fin buds in 5-day-old fugu embryos (Figs. 4a–b) as it is in late stage zebrafish embryos (Ng et al., 2002), showing that the gene we isolated is expressed in a pattern expected for *Fgf10*. We could not, however, detect any *Fgf10* expression around the pelvic fin forming region in fugu embryos at any stage examined (Fig. 4c). These results show that expression of *Fgf10* is not activated in the pelvic forming region of fugu. Because neither *Shh* nor *Fgf10* are expressed in the fugu pelvis-forming region, we conclude that the mechanism for pelvic fin bud outgrowth does not function in fugu.

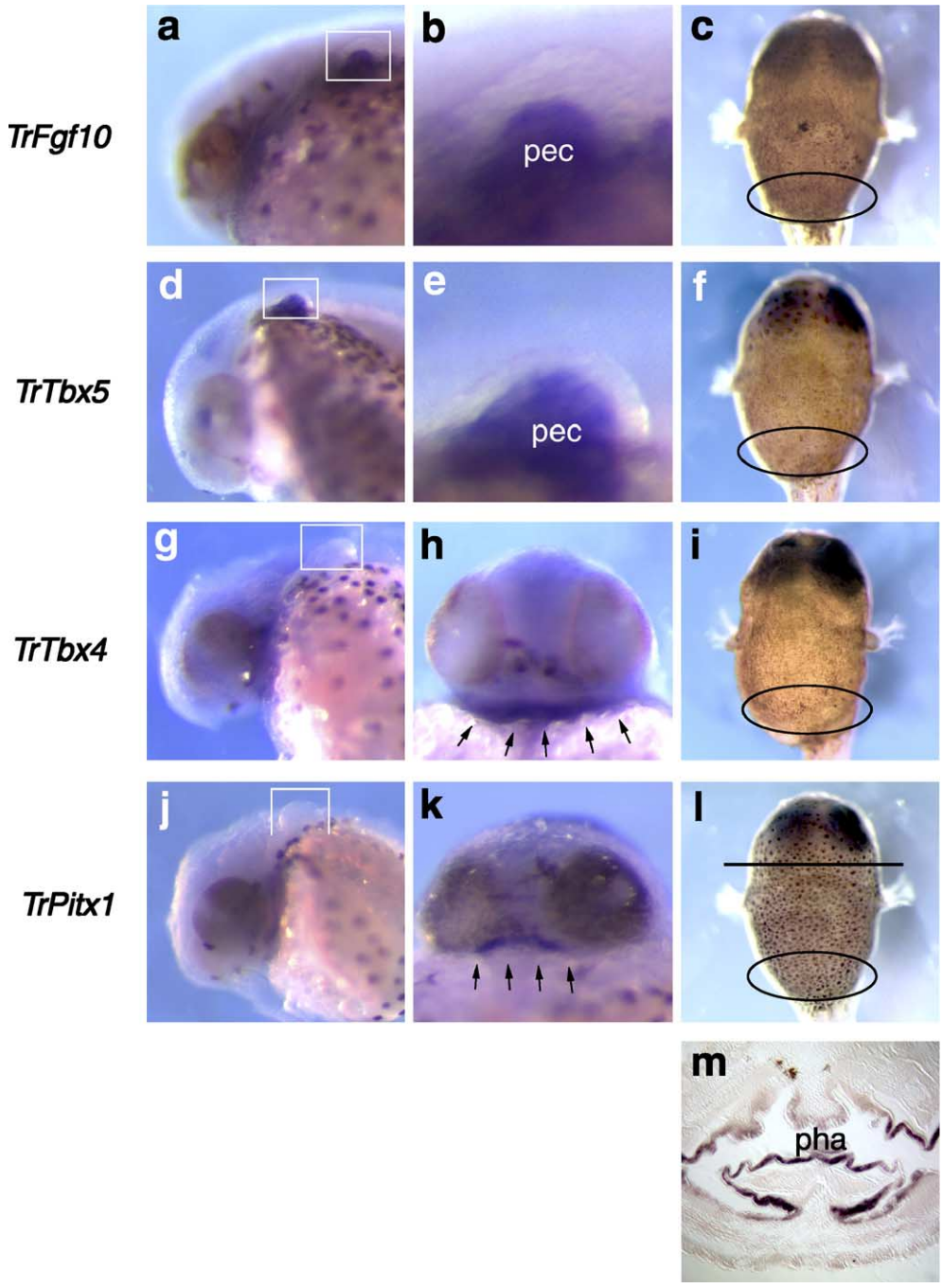

Fig. 4. Expression pattern of genes involved in initiation of fin/limb development in fugu. (a–c) *TrFgf10* expression. (a) Lateral view of 5-day-old larva. (b) Detail of pectoral fin region in (a). Note *TrFgf10* is strongly expressed in pectoral fin buds. (c) Ventral view of 28-day-old larva. No transcripts are detected around pelvic fin forming region (circled). (d–e) *TrTbx5* expression. (d) Lateral view of 5-days old larva. (e) Detail of pectoral fin bud region in (d). Transcripts in pectoral fin buds. (f) Ventral view of 28-day-old larva. No *TrTbx5* transcripts detected in pelvic region (circled). (g–i) *TrTbx4* expression. (g) Lateral view of 5-day-old larva. Note *TrTbx4* transcripts absent from pectoral fin bud region. (h) Frontal view of 5-day-old larva. Transcripts in mandibular region (arrows). (i) Ventral view of 28-day-old larva. No *TrTbx4* transcripts in pelvic region (circled). (j–m) *TrPitx1* expression. (j) Lateral view of 5-day-old larva. *TrPitx1* transcripts not detected in pectoral fin bud. (k) Arrows indicates *TrPitx1* transcripts in mandibular region. (l) Ventral view of 28-day-old larva. *TrPitx1* transcripts are not detected in pelvic region (circled). (m) Transverse section of 28 days larva through the mouth, as shown in (l). *TrPitx1* is expressed in the epithelium of the mouth. Abbreviations: pec, pectoral fin bud; pha, pharynx.

Because we detected no evidence for the genetic control system for pelvic bud outgrowth in fugu, we investigated the hypothesis that bud initiation phase is normal by examining two T-box containing transcription factors important for limb initiation. *Tbx5* and *Tbx4* are involved in the activation of *Fgf10* expression in the limb-forming region of chick and mouse embryos (see Fig. 1) (Agarwal et al., 2003; Rallis et al., 2003; Takeuchi et al., 2003; Ng et al., 2002). We isolated PCR fragments of fugu *Tbx5* and *Tbx4* and performed in situ hybridization experiments on fugu embryos of various ages.

TrTbx4 is located on fugu Scaffold_672, which also has fugu orthologs of the human genes *BRIP1* (SINFRUT00000139789), *PPM1D* (SINFRUT00000139798), and *APPBP2* (SINFRUT00000139803), which are adjacent in the human genome, and only one gene (*BCAS3*) in this human segment is missing from the fugu contig; this conserved synteny information supports the assignment of orthology for *Tbx4*. Likewise, we identified a fugu *Tbx5* ortholog as SINFRUT00000155903 on Scaffold_218, which is adjacent to SINFRUT00000155902, the fugu ortholog of human *DDX54*, which is six genes distant from *TBX5* in the human genome, supporting the orthology assignment. *Tbx5* is associated with vertebrate forelimb development while *Tbx4* is associated with hindlimb development (reviewed in Tanaka and Tickle, in press). We found abundant transcripts of *Tbx5* in the pectoral fin buds of five-day old fugu embryos (Figs. 4d–e). After hatching, *Tbx5* expression continued in pectoral fins as well as dorsally in the eye, but become weaker and finally disappeared. At no point was *Tbx5* expression observed in the pelvic region (circled in Fig. 4f). Similar *Tbx5* expression patterns have been reported in zebrafish (Begemann and Ingham, 2000; Ng et al., 2002; Ruvinsky et al., 2000; Tamura et al., 1999), dogfish (Tanaka et al., 2002), chick (Gibson-Brown et al., 1998a; Isaac et al., 1998; Logan et al., 1998; Ohuchi et al., 1998), and mouse (Agarwal et al., 2003; Gibson-Brown et al., 1996), suggesting that *Tbx5* plays a similar role in all gnathostome vertebrates. Zebrafish homozygous for a mutation in *tbx5* lack pectoral fins completely, and morpholino abrogation of *tbx5* activity results in the deformations of pectoral fins (Ahn et al., 2002; Garrity et al., 2002; Ng et al., 2002). Furthermore, forelimbs do not initiate their development in mice in which *Tbx5* is conditionally inactivated (Rallis et al., 2003). Therefore, *Tbx5* is important for initiation of pectoral fin bud and forelimb in fish, and these signals for initiation of pectoral fin buds are activated normally during fugu development.

While *Tbx5* is important for the initiation phase of pectoral appendage development, *Tbx4* is required for the initiation phase of pelvis development because inhibiting *Tbx4* transcription in chick embryos deletes the hindlimb (Takeuchi et al., 2003), and mice lacking *Tbx4* function fail to develop hindlimbs (Naiche and Papaioannou, 2003). In situ hybridization experiments on fugu showed that *Tbx4* was expressed in the developing mouth of fugu embryos (arrows in Fig. 4h), providing a positive control for probe efficacy. In contrast, *Tbx4* transcripts were not detected in fugu pectoral fin buds (Fig. 4g), nor were they observed in the pelvic region of fugu embryos at any stage examined (Fig. 4i). This contrasts to zebrafish, where pelvic fin bud initiation is detected by 3 weeks (Grandel and Schulte-Merker, 1998), and *tbx4* expression begins in pelvic fins before 4 weeks (Ruvinsky et al., 2000; Tamura et al., 1999). We conclude that *Tbx4* is not activated normally in fugu embryos, showing that the initiation phase does not happen normally in fugu pelvic fin development.

The hindlimb-specific expression of *Tbx4* is activated by the homeobox-containing transcription factor *Pitx1* (Logan and Tabin, 1999). In *Pitx1*$^{-/-}$ mouse embryos, *Tbx4* expression in hindlimb buds is reduced (Lanctot et al., 1999; Marcil et al., 2003; Szeto et al., 1999), and the resulting hindlimbs are malformed. We identified *Pitx1* in fugu as SINFRUT00000142215, and found that it lies on Scaffold_527, adjacent to fugu orthologs of human loci *DCOHM*, *C5orf14*, *FLJ37562*, and *DDX46*. These human loci are adjacent to each other, and except for one gene (*CATSPER3*) which is apparently absent from fugu, they are in the same order and transcribed in the same direction in fugu and human. With this evidence confirming orthology assignments, we designed primers to isolate the fugu *Pitx1* gene and examined its expression pattern. In fugu, as in mouse, *Pitx1* is not expressed in the pectoral appendage buds (Fig. 4j), but transcripts are abundant in the developing mouth (arrows in Fig. 4k). In the pelvic region of fugu embryos, *Pitx1* expression did not appear at any developmental stage up to 5-week-old larvae, although *Pitx1* was expressed in the epidermis around the pharynx in fugu larvae (Fig. 4m). These results rule out the hypothesis that fugu pelvic fin failure occurs after the *Pitx1* activation step of the initiation stage. Therefore, we conclude that pelvic loss in fugu is due either to lack of *Pitx1*-activation signals during the pelvic initiation phase, or to the absence of pelvic fin positional cues. This conclusion drove us to explore the mechanisms providing pelvic fin position in fugu larvae.

Bud positioning

Hox genes specify axial pattern during embryonic development, and in animals with different numbers of vertebrae, *Hox* expression in the paraxial mesoderm correlates with vertebral identity (Burke et al., 1995). *Hox* genes are also involved in the regionalization of the lateral plate mesoderm into prospective forelimb, prospective flank between the limbs, and prospective hindlimb positions (Cohn et al., 1997), and altered *Hox* gene expression in python embryos correlates with loss of axial positions appropriate for pectoral appendages. To test the hypothesis that changes in *Hox* gene expression in the paraxial mesoderm and lateral plate mesoderm correlate with the loss of pelvic fins in fugu in analogy with the loss of forelimb axial position appropriate for forelimbs in snakes (Cohn and Tickle, 1999), we examined the expression pattern of a few key *Hox* genes.

The expression boundary of *Hoxc6* in the paraxial mesoderm in tetrapod embryos seems to be related to the position of the forelimb and the anatomical boundary between the neck and the thorax (Burke et al., 1995). Likewise, zebrafish pectoral fins arise at the level of the anterior limit of *hoxc6a* and *hoxc6b* expression at the somite 4/5 border (Burke et al., 1995; Prince et al., 1998; Molven

et al., 1990; Morin-Kensicki et al., 2002). We therefore isolated *Hoxc6a* from stickleback and fugu (both of which, in contrast to zebrafish, have a single copy of the *Hoxc* complex (Amores et al., 1998, 2004; Aparicio et al., 1997, 2002; Venkatesh et al., 2000; A. Amores and W. Cresko, unpublished). We then examined *Hoxc6a* expression patterns with respect to the position of pectoral and pelvic fins. In lateral plate mesoderm of 2-day-old stickleback embryos, *Hoxc6* transcripts are abundant around the presumptive pectoral fin bud region (arrows in Figs. 5a–b) and the anterior border of *Hoxc6* expression in paraxial mesoderm is aligned with the presumptive pectoral fin bud region (Figs. 5a–b), corroborating earlier work (Ahn, 1998; Ahn and Gibson, 1999). In 3-day-old stickleback embryos, pectoral fin buds expressed *Hoxc6* more intensely in the anterior fin bud than the posterior bud (arrows in Figs. 5c–d), mimicking the situation in pectoral fin buds of zebrafish (Molven et al., 1990). Transcripts of *Hoxc6* are also abundant in the anterior region of the pectoral fin buds of 4-day-old fugu embryos. Furthermore, the anterior border of *Hoxc6* expression in the paraxial mesoderm is aligned with the pectoral fin buds as in other vertebrates (Figs. 5e–f). These experiments show that axial expression of *Hoxc6* is appropriate for defining the position of pectoral fins along the anterior–posterior axis in fugu and suggest that the usual vertebrate mechanisms that position pectoral fins along the body axis are conserved in fugu and stickleback.

Hox genes also help specify the position of pelvic appendages in vertebrates. Prior to the initiation of limbs in chick embryos, the anterior expression border of *Hoxd9* in the lateral plate mesoderm lies at the flank–wing junction. When limb development initiates, the anterior boundary of *Hoxd9* expression shifts to the anterior limit of the wing bud. Subsequently, expression of *Hoxd9* in the flank down-regulates, and then disappears (Cohn et al., 1997; summarized here in Fig. 7). In chick embryos, pelvic appendage buds first appear at about the time pectoral appendage buds begin to develop. Although in tetrapods, pectoral and pelvic appendages arise synchronously, in teleost fishes, pelvic appendages develop later than pectoral appendages. For example, pectoral fin buds of zebrafish initiate their development by 26 h post-fertilization, while pelvic fin buds develop just before 3 weeks post-fertilization midway through metamorphosis (Grandel and Schulte-Merker, 1998). To test the phylogenetic stability of the correlation between *Hoxd9* expression and pelvic fin positioning, and to test the hypothesis that changes of *Hoxd9* expression do not underlie pelvic fin loss in fugu, we isolated *Hoxd9a* from fugu and stickleback and examined expression patterns during development.

In 2-day-old stickleback embryos, *Hoxd9* expression was detected throughout the lateral plate mesoderm from the pectoral fin forming region to the tail region (Figs. 6a–b). By 3 days post-fertilization, *Hoxd9* transcripts in lateral plate mesoderm accumulated at the pectoral fin bud level and disappeared from the caudal part of the body wall (Figs. 6c–d). In contrast, in chick embryos, *Hoxd9* expression disappears only from the flank between the wing and leg bud axial levels. Strikingly, in 21-day-old anadromous stickleback embryos, the expression of *Hoxd9* reappears in the body wall between the pectoral and pelvic fin buds,

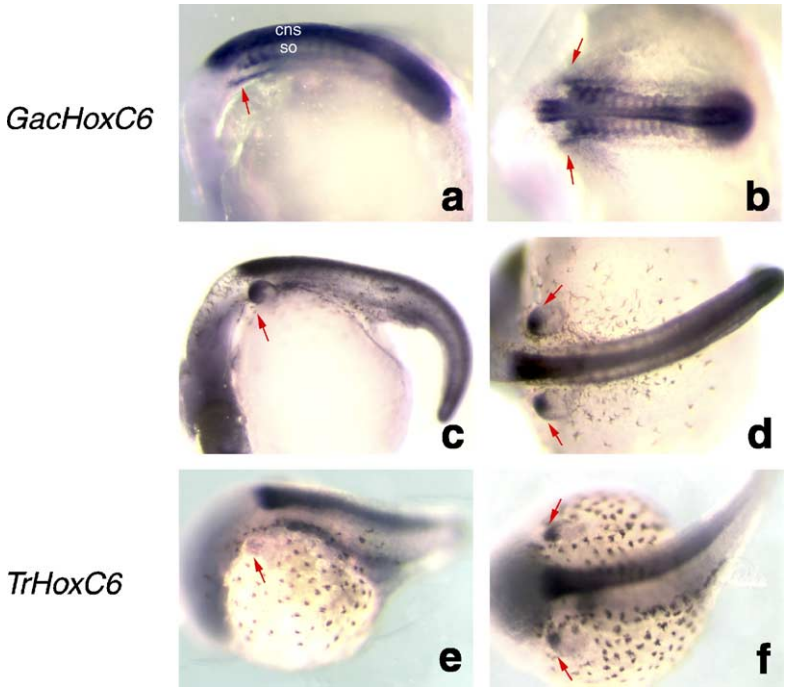

Fig. 5. Expression pattern of *Hoxc6* in stickleback and fugu. (a–d) *GacHoxc6* expression of *G. aculeatus* (stickleback) larva. (a–b) 2-day-old larva. (c–d) 3-day-old larva. (a, c) Lateral view. (b, d) Dorsal view. (c–d) *TrHoxc6* expression of 4-day-old fugu larva. (e) Lateral view. (f) Dorsal view. Red arrows indicate pectoral fin region. Abbreviations: cns, central nervous system; so, somites.

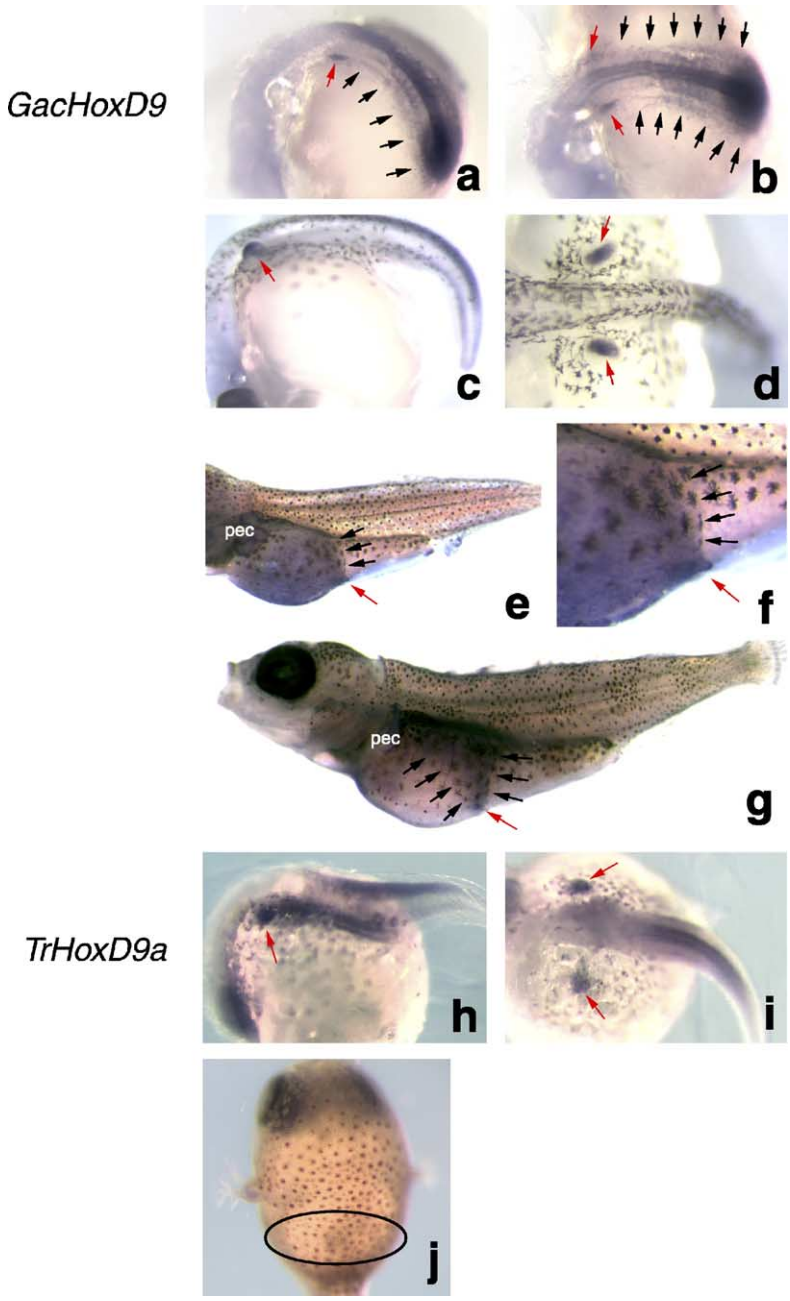

Fig. 6. Expression pattern of *Hoxd9* in stickleback and fugu. (a–g) *GacHoxd9* expression of *G. aculeatus* larva. (a–b) 2-day-old larva. Transcripts in lateral plate mesoderm (black arrows). Red arrows indicate *GacHoxd9* expression in pectoral fin region. (c–d) 3-day-old larva. *GacHoxd9* is expressed in the pectoral fin buds (red arrows). (e) 21-day-old larva. Red arrow indicates pelvic fin bud. Black arrows indicate expression border of *GacHoxd9* in lateral plate mesoderm. (f) Detail of pelvic fin bud region in (e). (g) 25-day-old larva. Red arrow indicates pelvic fin bud. Black arrows indicate expression border of *GacHoxd9* in lateral plate mesoderm. (a, c, e, g) Lateral view. (b, d) Dorsal view. (h–j) *TrHoxd9a* expression of fugu larva. (h) Lateral view of 4-day-old larva. Transcripts are abundant in pectoral fin bud (red arrow). (i) Dorsal view of 4-day-old larva. *TrHoxd9a* is expressed in pectoral fin buds (red arrows). (j) Ventral view of 28-day-old larva. No *TrHoxd9a* transcripts in pelvic region (circled).

which were positioned at the posterior border of the *Hoxd9* expression domain (arrows in Figs. 6e–f). Subsequently, *Hoxd9* expression in the flank between the pectoral fins and pelvic fin buds became faint, and transcripts persisted only near the pelvic fin buds (arrows in Fig. 6g). We conclude that *Hoxd9* expression in stickleback correlates not only with pectoral fin positioning, but also with pelvic fin positioning.

To examine whether *Hoxd9* defines the pelvic forming region in pufferfish as it does in stickleback, we examined *Hoxd9* expression in fugu embryos. Unlike zebrafish (Amores et al., 1998), fugu has two copies of *Hoxd9–Hoxd9a* and *Hoxd9b* (Amores et al., 2004; Aparicio et al., 2002). In 4-day-old fugu embryos, expression of *Hoxd9a* appeared in pectoral fin buds as in stickleback embryos (Figs. 6h–i), but *Hoxd9b* expression was hardly detected in the lateral plate

mesoderm at any stage from 4 days to 28 days post-fertilization (data not shown). Although *Hoxd9a* transcripts were abundant in pectoral fin bud mesoderm at early stages of fugu embryos, and in the pelvic region of anadromous stickleback (Fig. 6g), no transcripts were detected around the pelvic fin forming region up to 5 weeks in fugu (circled in Fig. 6j). We conclude that the lack of *Hoxd9a* expression in the body wall at the time of pelvic fin initiation correlates with the absence of the pelvic fin in fugu.

Conclusions

This report describes comparative genetic analyses investigating several hypotheses for the evolution of genetic regulatory systems controlling development of pelvic appendages. We isolated from the genomes of the pufferfish fugu, which lacks a pelvic apparatus, and anadromous threespine stickleback, which has a robust pelvis, genes thought to control pelvic appendage development in tetrapods. Results showed no evidence for pelvis skeletogenesis (Alcian blue staining), for the genetic circuitry controlling outgrowth of the pelvic fin bud (*Shh* and *Fgf10* expression), or for pelvis-specific fin bud initiation mechanisms (*Tbx4*, *Tbx5*, *Pitx1* expression) in fugu embryos and larvae through metamorphosis. These results show that evolutionary mechanisms have led to obstruction of the conserved developmental regulatory system for pelvic apparatus development either very early in the initiation phase, or in the positioning phase of the appendage developmental pathway (Fig. 7).

Experiments examining the positioning phase of appendage development revealed similarities in *Hox* positioning signals for pectoral appendages in pufferfish, stickleback, and tetrapods. Likewise, for the pelvic-level *Hox* positioning signals, anadromous stickleback with pelvic appendages maintained *Hoxd9* expression in the pelvic apparatus. Stickleback differed from tetrapods, however, by the maintenance of *Hoxd9* expression in the flank between the pectoral and pelvic apparatus, while in tetrapods, this expression domain disappears in the outgrowth phase of bud development. We hypothesize that the maintenance of the trunk expression domain of *Hoxd9* in stickleback may be functionally related to a major synapomorphy of the Percomorpha, the taxon including pufferfish and stickleback: these fish share the rostral positioning of the pelvic apparatus, which moves during development close to, or in some cases even rostral to the pectoral fins (Nelson, 1994). One imagines that the flank expression domain of *Hoxd9* would shrink as the pelvic apparatus moves forward.

In contrast to stickleback, the *Hoxd9* expression domain of fugu does not develop in the trunk or in the region corresponding to the stickleback pelvis. This expression loss supports the conclusion that pufferfish lack a pelvis because they are deficient in axial positioning cues appropriate for pelvis, although we cannot rule out the possibility that the novel *Hoxd9* expression in stickleback body wall is a character unique to the stickleback lineage. This explanation would be analogous to the mechanism leading to the absence of pectoral axial patterning cues in python snakes (Cohn and Tickle, 1999). We do not know if the mutational event that results in the changed *Hoxd9* expression pattern occurred in a

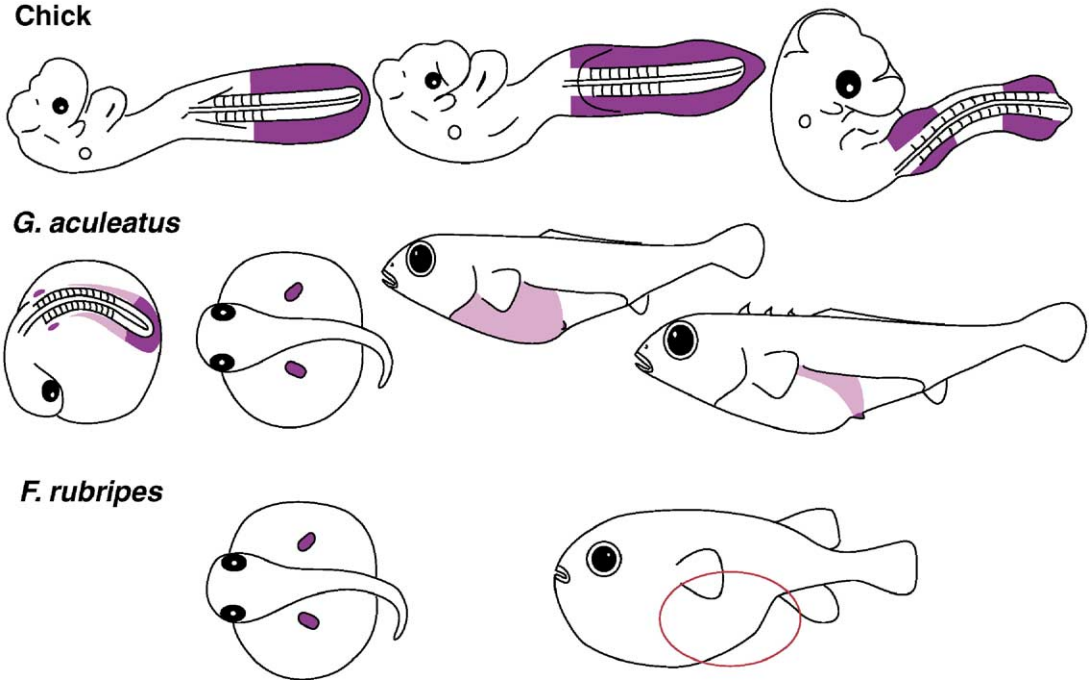

Fig. 7. Diagrammatic summary of *Hoxd9* expression in lateral plate mesoderm of chick, *G. aculeatus* and *T. rubripes* embryos. Expression of *Hoxd9* in chick according to Cohn et al. (1997). Abbreviations: pec, pectoral fins; pel, pelvic fins; w, wing region; l, leg region.

cis-acting regulatory element of *Hoxd9*, or in an upstream factor that regulates *Hoxd9* expression in this region of the body axis. Unfortunately, fugu embryos are not currently amenable to laboratory manipulations to test these hypotheses. Transgenesis experiments in zebrafish and stickleback, however, are possible and should allow us to test whether fugu *Hoxd9* lacks cis-acting regulatory elements responsible for flank and pelvic expression of *Hoxd9* present in the orthologous zebrafish and stickleback genes.

The *Hoxd9* knockout mouse has defective forelimbs but not hindlimbs (Fromental-Ramain et al., 1996). *Hoxd9* knockout mice may lack a pelvic phenotype because *Hoxb9* and *Hoxc9* may compensate for the role of *Hoxd9* in limb positioning in tetrapods. This compensation would not be expected for forelimb patterning in tetrapods because, in contrast to the pelvic limb, *Hoxd9* is expressed in the forelimb during the limb positioning phase in the absence of the other three *Hox9* group genes.

The genetic mechanism for the evolution of pelvic reduction in fresh water stickleback from ancestral anadromous animals with formidable pelvic spines has been investigated recently. Results showed that some populations have a reduced or absent pelvis due to a single Mendelian factor that maps in or near the *Pitx1* gene, a factor in pelvic bud initiation that is expressed in the pelvic region in anadromous but not fresh water stickleback (Cole et al., 2003; Cresko et al., 2004; Shapiro et al., 2004). Those results in combination with the experiments reported here suggest that evolutionary loss of the pelvis in stickleback and pufferfish is associated with aberrations in different phases of the appendage developmental program, positioning in the case of pufferfish and initiation in the case of stickleback. Thus, a similar phenotype in related taxa has resulted from mutations affecting different phases of the same developmental pathway.

When did the pufferfish lose its pelvis? Lineages in the teleost Order Tetraodontiformes have experienced pelvic loss at least twice: at least once in the Suborder Sclerodermi (in the lineage leading to boxfish and cowfish), and at least once in the Suborder Gymnodontes (Santini and Tyler, 2003). The Gymnodontes includes the three-tooth pufferfish (Infraorder Triodontoideo), which has a pelvic apparatus, and the Infraorder Tetraodontoideo, a clade including four-tooth pufferfish such as fugu, porcupine fish, and ocean sunfish, all of which lack a pelvis (Santini and Tyler, 2003). This puts the loss of the pelvis in the pufferfish lineage before 35 million years ago, and perhaps before 53 million years ago. In contrast, extant circumarctic lake and stream populations of stickleback that lack a pelvic appendage evolved much more recently, since the recession of the glaciers in the last 14,000 years (Bell, 1987; Bell et al., 1993; McPhail, 1992), although fossil forms with reduced skeletons are known from 30,000 years ago or older (Bell and Legendre, 1987; Swift, 1989). Fresh water stickleback may evolve pelvis loss by natural selection in the absence of piscivorous fish and presence of predatory insects and/or the expense of bone-building in calcium-poor fresh waters (Bell et al., 1993; Giles, 1983; Nelson and Reimchen, 1983; Reimchen, 1988; Swift, 1989). Selective forces for the loss of pelvic fins in pufferfish is unknown, but the presence of a rostral pelvis that articulates with the head skeleton may inhibit the rapid ingestion of water into the digestive tract associated with puffing.

Neither the stickleback nor the pufferfish investigations show that mutations in initiation or positioning, respectively, were the original genetic variations upon which selection or drift acted to reduce pelvic size. One could imagine that mutations could initially have occurred blocking any of the components of the program, and that as generations passed, mutations blocking earlier phases could have become fixed in descendent populations due either to selection or drift, resulting in the situation we see today. It may be a general rule that downstream elements of a developmental pathway may become mutated early in the evolution of the loss of a feature, and upstream elements become mutated later.

These results for fugu, taken with other results for stickleback and pythons, show that the common phenotype of pelvic reduction can occur due to abrogation of different phases in the conserved genetic pathway for appendage development, either at the outgrowth phase for pythons (Cohn and Tickle, 1999), the initiation phase for independently evolved populations of stickleback in Scotland, Alaska, Canada, or Iceland (Cole et al., 2003; Cresko et al., 2004; Shapiro et al., 2004), or in the positioning phase in fugu. Whether the phase of development that is blocked in independent cases of the evolution of similar phenotypes is determined by lineage-specific developmental constraints or is stochastic, depending on which genes in the conserved regulatory pathway happen to become mutated first is not yet known.

Acknowledgments

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