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Fgf16 is essential for pectoral fin bud formation in zebrafish

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Abstract

Zebrafish pectoral fin bud formation is an excellent model for studying morphogenesis. Fibroblast growth factors (Fgfs) and sonic hedgehog (shh) are essential for pectoral fin bud formation. We found that *Fgf16* was expressed in the apical ectodermal ridge (AER) of fin buds. A knockdown of Fgf16 function resulted in no fin bud outgrowth. Fgf16 is required for cell proliferation and differentiation in the mesenchyme and the AER of the fin buds, respectively. Fgf16 functions downstream of Fgf10, a mesenchymal factor, signaling to induce the expression of *Fgf4* and *Fgf8* in the AER. *Fgf16* in the AER and *shh* in the zone of polarizing activity (ZPA) interact to induce and/or maintain each other's expression. These findings have revealed that Fgf16, a newly identified AER factor, plays a crucial role in pectoral fin bud outgrowth by mediating the interactions of AER-mesenchyme and AER-ZPA.

Keywords: Fgf; Fgf16; Zebrafish; Fin; Apical ectodermal ridge; Development; Morphogenesis

Vertebrate limb/fin bud formation is considered an excellent model for studying morphogenesis. Numerous factors have been implicated in the processes of limb/fin bud initiation and outgrowth in vertebrates [1]. Fgfs function as key secreted signaling molecules in morphogenesis including limb/fin bud formation. The vertebrate Fgf family comprises 22 members [2]. Evidence has been accumulating that at least three members of the Fgf family function as key intercellular signaling molecules in the processes of vertebrate limb bud formation. *Fgf10* is expressed in the limb bud-forming region of the mesenchyme [3]. *Fgf10* knockout mice have no limbs, indicating that Fgf10 is absolutely required for limb bud outgrowth [4,5]. In contrast, both *Fgf4* and *Fgf8* are expressed in the apical ectodermal ridge (AER). Conditional *Fgf4* knockout mice have normal limbs [6,7]. Limb development in conditional *Fgf8* knockout mice is abnormal but limb bud outgrowth continues [8]; however, limb development in *Fgf4*/*Fgf8* double knockout mice is severely impaired [9,10]. These results indicate that both Fgf4 and Fgf8 have crucial but redundant functions in limb development.

Zebrafish pectoral fin buds are homologous to tetrapod forelimb buds [11]. Fgf signaling also plays crucial roles in pectoral fin bud formation. *Fgf10* is expressed in the mesenchyme of the pectoral fin bud-forming region and is required for pectoral fin bud outgrowth [13]. *Fgf4* and *Fgf8* are also expressed in the AER of pectoral fin buds and are expected to play roles in the pectoral fin bud formation [12]. *Fgf24* in the mesenchyme of pectoral fin primordia induces the expression of *Fgf10* in the mesenchyme [14,15]. However, a human or mouse ortholog of zebrafish *Fgf24* has not been identified in their genomes.

Fgf16 was originally identified from rat embryos [16,17]. However, the role of Fgf16 in morphogenesis remains to be elucidated. The zebrafish is a useful model for elucidation of the functions of genes in vertebrates in vivo. We identified zebrafish *Fgf16* and examined its role. Zebrafish *Fgf16* was expressed in several discrete regions of embryos including the AER of pectoral fin buds. Fgf16 was found to be an AER factor crucial for pectoral fin bud outgrowth.

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Materials and methods

Fish maintenance. Zebrafish embryos were obtained by natural spawning and cultured at 28.5 °C in Zebrafish Ringer's solution.

Isolation and characterization of zebrafish Fgf16 cDNA. A BLAST (Basic Local Alignment Search Tool) search of zebrafish genomic DNA sequences with the amino acid sequence of human *FGF16* identified zebrafish *Fgf16* genomic DNA. The full-length cDNA was amplified by PCR with the cDNA as a template and cloned into the pGEM-T DNA vector (Promega, Madison, WI). The apparent evolutionary relationships between zebrafish Fgf16 and members of the human FGF family were examined using Clustal W. The positions of zebrafish *Fgf16* and *atrx* on chromosomal fragments were obtained from the Ensembl Zebrafish Genome Browser. The map positions of human *FGF16* and *ATRX* were obtained from LocusLink.

Whole mount in situ hybridization. Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using T7 or SP6 RNA polymerase. Whole mount in situ hybridization was performed as described [19]. A 0.6-kb *Fgf16* probe was synthesized using the full-length cDNA-containing plasmid. Other probes used were zebrafish *dlx2* [20], *shh* [21], *Fgf10* [13], *Fgf8* [22], *Fgf4* [12], and *pax2.1* [23].

Gene knockdown by morpholinos. Morpholino oligonucleotides (MOs) were synthesized by Gene-Tools, LLC (Corvallis, OR). MOs were diluted in Danieau buffer [24]. For the sequences of MOs and the injection of MOs into embryos. *Fgf16* MO, the sequence of which corresponds to the coding region including the initiation codon, 5′-GAGAAATCCAGCCACCTCT GCCATG-3′; five-base mismatched (control) *Fgf16* MO, 5′-GAcAAATg CAGCgACCTgTGCgATG-3′; splice-site-targeted *Fgf16* MO (E2I2), the sequence of which corresponds to that between the second exon and intron of the coding region, 5′-CTA TGGGTCGGTAAGTCAAACTCCT-3′; *Fgf10* MO, 5′-CCTTAGTCA CTTTCCATTTGCACAT-3′; *Fgf8* MO, 5′-GAGTCTCATGTTTATAG CCTCAGTA-3′; *Fgf4* MO, 5′-GCTACC GTTTTTCTCTATGCTTGAG-3′. Lowercase letters in control *Fgf16* MO indicate mismatches with nucleotides in *Fgf16* MO. MOs were diluted in Danieau buffer [24]. *Fgf16* MO (15 μg/μl), control *Fgf16* MO (15 μg/μl) or splice-site-targeted *Fgf16* MO(E2I2) (14 μg/μl) was injected at a volume of 0.30–0.40 nl into two-cell embryos. *Fgf10* MO (15 μg/μl) was injected at a volume of 0.30–0.40 nl into two-cell embryos. *Fgf8* MO (20 μg/μl) was injected at a volume of 0.20–0.25 nl into two-cell embryos. *Fgf4* MO (15 μg/μl) was injected at a volume of 0.65–0.70 nl into two-cell embryos.

Detection of phosphohistone H3. Zebrafish embryos were fixed in 4% paraformaldehyde/phosphate-buffered saline at 4 °C overnight. Phosphohistone H3 in fixed embryos was detected by immunohistochemistry using anti-H3P rabbit polyclonal antibody (1:100) (Upstate Biotechnology, Lake Placid, NY) and HRP-conjugated anti-rabbit IgG (1:200) (Vector Laboratory, Burlingame, CA). The immunohistochemistry was performed essentially according to a published protocol [25]. For cell counts, sections of stained embryos were prepared as described [28].

Cyclopamine treatment. Cyclopamine (Toronto Researh Chemical, North York, Canada) was dissolved at 10 mM in ethanol. Embryos, in their chorions, were incubated in cyclopamine diluted to 200 μM in Zebrafish Ringer's solution from 5 to 36 hpf in the dark. They were fixed in 4% paraformaldehyde [26].

Results and discussion

Identification of zebrafish Fgf16

Among vertebrates, the amino acid sequences of most orthologous Fgfs are highly conserved [2]. We identified zebrafish *Fgf* genes by BLAST-searching the zebrafish genomic sequences with the amino acid sequences of human FGFs. Then, we isolated their full-length cDNAs from zebrafish embryos. One of the cDNAs encodes a protein of 203 amino acids that is highly homologous (80% amino acid identity) to human FGF16 (data not shown). Phylogenetic analysis also indicates that the protein is most closely related to human FGF16 (Fig. 1A). Human *FGF16* is closely linked with the α thalassemia/mental retardation syndrome X-linked homolog gene (*ATRX*) on chromosome X at q21.1 [2]. The zebrafish gene was also found to be closely linked with *Atrx* in the genome (Fig. 1B). We conclude that the gene is zebrafish *Fgf16* (GenBank Accession No. AB201764).

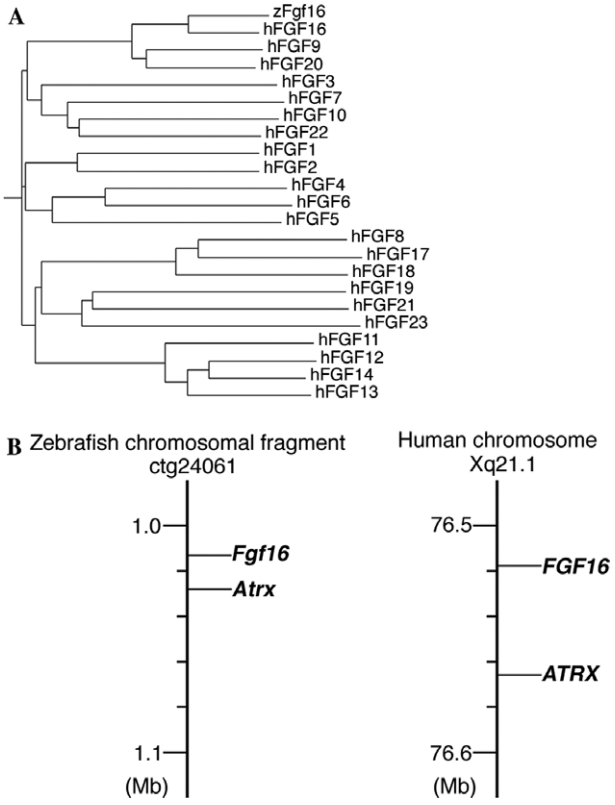

Fig. 1. Molecular analysis of zebrafish *Fgf16*. (A) Phylogenetic tree comparing zFgf16 with hFGFs. The apparent evolutionary relationships of zebrafish Fgf16 with members of the human FGF family were examined using Clustal W. (B) Syntenic relationship between zebrafish chromosomal fragment ctg24061 and human chromosome Xq21.1. The *zFgf16* and *hFGF16* genes are closely linked to the zebrafish *Atrx* and human *ATRX* genes, respectively. Mb, megabase.

Expression of Fgf16 in zebrafish embryos

We examined the spatiotemporal expression of *Fgf16* in zebrafish embryos at different developmental stages (12–36 h post-fertilization (hpf)) by whole mount in situ hybridization. *Fgf16* was not clearly detected at 12 and 14 hpf (data not shown). At 18 and 24 hpf, *Fgf16* was expressed in the otic vesicle, telencephalon, and caudal fin (Fig. 2A and B). At 30 and 36 hpf, *Fgf16* was expressed in the otic vesicle, caudal fin, branchial arch, pectoral fin bud, and pituitary gland (Fig. 2C and D).

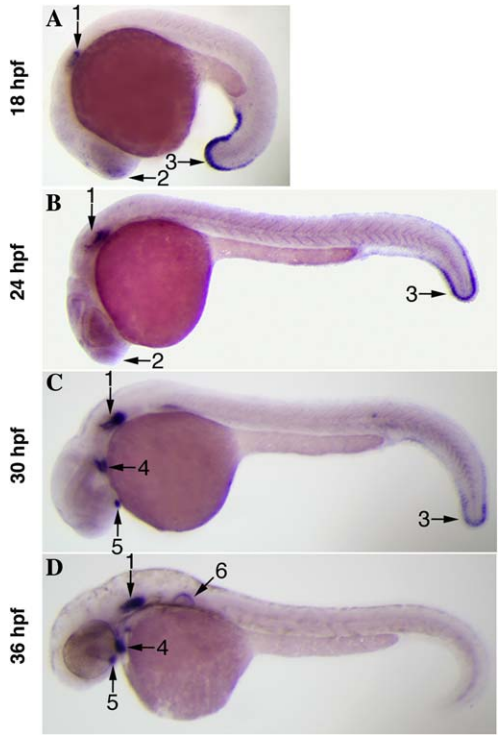

Fig. 2. Expression pattern of *Fgf16* during zebrafish embryonic development. At 18 and 24 hpf, *Fgf16* was expressed in the otic vesicle, telencephalon, and caudal fin (A,B). At 36 hpf, *Fgf16* was also expressed in the otic vesicle, caudal fin, branchial arch, pectoral fin buds, and pituitary gland (C,D). Arrows indicate the otic vesicle (1), the telencephalon (2), the caudal fin (3), the branchial arch (4), the pituitary gland (5), and the pectoral fin bud (6).

Inhibition of Fgf16 functions results in defects in formation of pectoral fins

Injections of antisense morpholino oligonucleotides (MOs) have been demonstrated to inhibit the corresponding gene functions in zebrafish embryos [24]. We injected *Fgf16* or five-base mismatched *Fgf16* (control) MO into zebrafish two-cell embryos. The *Fgf16* MO sequence (25 bases) corresponds to the coding region including the initiation codon. The *Fgf16* MO-injected embryos at 5 days post-fertilization (dpf) had no pectoral fins (Fig. 3Ab) ($n = 104$, death 27/104, no fins 45/104, partial fins 28/104, and normal 4/104). In contrast, the control MO-injected embryos developed normally (Fig. 3Aa) ($n = 108$, death 4/108, no fins 0/108, partial fins 9/108, and normal 95/108). We also injected splice-site-targeted *Fgf16* MO (E2I2), the sequence (25 bases) of which corresponds to that between the second exon and intron of the coding region, into two-cell embryos (Fig. 4A). The phenotype of *Fgf16* MO (E2I2) MO-injected embryos at 5 dpf was quite similar to that of *Fgf16* MO-injected embryos including the absence of pectoral fins (Fig. 3Ac) ($n = 32$, death 3/32, no fins 13/32, partial fins 10/32, and normal 6/32). RNA was isolated from wild-type embryos and splice-site-targeted *Fgf16* MO (E2I2)-injected embryos with no fins at 48 hpf. *Fgf16* cDNA was amplified from the RNA by RT-PCR using primers (P2 and P3) the sequences of which correspond to those of exons two and three (Fig. 4A). The amplified cDNA from *Fgf16* MO (E2I2)-injected embryos was slightly shorter than the wild-type cDNA (285 bp) (Fig. 4B). The wild-type cDNA was subjected to normal splicing. In contrast, the cDNA from *Fgf16* MO (E2I2)-injected embryos was subjected to abnormal splicing, resulting in a truncated translation product (Fig. 4C). These results indicate that Fgf16 is crucial for the formation of pectoral fins.

Pectoral fin formation at different developmental stages was also observed. In control MO-injected embryos, the bud was a shallow dome at 36 hpf and further grew at 42 hpf. At 48 hpf, the bud curved posteriorly and tapered (Fig. 3Ba–e). In contrast, no bud was observed at 36 hpf in the *Fgf16* MO-injected embryos. At 42 hpf, the bud was observed as a shallow dome. Even at 48 and 72 hpf, the buds remained as shallow domes (Fig. 3Bf–j). These results indicate that the outgrowth but not the initiation was greatly impaired in the Fgf16 knockdown fin buds.

Fgf16 is required for expression of dlx2, Fgf16, Fgf8, and Fgf4 in AER and shh in ZPA but not for Fgf10 in mesenchyme

dlx2 is a marker for the AER [20]. We examined the expression of *dlx2* in zebrafish fin buds by whole mount in situ hybridization. The expression of *dlx2* was detected in the AER of fin buds of control MO-injected embryos at 42 hpf (Fig. 3Ca). In contrast, the expression of *dlx2* was not detected in *Fgf16* knockdown pectoral fin buds (Fig. 3Cg). These results indicate that differentiation of the AER in the Fgf16 knockdown fin buds was greatly impaired.

We examined the expression of *Fgf16* in pectoral fin buds at 36 hpf by whole mount in situ hybridization. *Fgf16* was also expressed in the AER (Fig. 5Aa and b). The expression of *Fgf16* was examined in *Fgf16* MO-injected embryos at 42 hpf. The expression of *Fgf16* was greatly repressed in the AER of *Fgf16*-knockdown embryos (Fig. 3Ch). This result was consistent with the finding that differentiation of the AER in the *Fgf16* knockdown fin buds was greatly impaired as described above.

Fgf10 expressed in the mesenchyme of pectoral fin buds is required for developing fin buds [13]. We examined the expression of *Fgf10* in pectoral fin buds of *Fgf16* MO-injected embryos. The expression of *Fgf10* was definitely detected in *Fgf16* knockdown fin buds at 42 hpf (Fig. 3Ci). *Fgf4* and *Fgf8* are expressed in the AER of pectoral fin buds [12]. We also examined the expression of *Fgf8* and *Fgf4* in the pectoral fin buds. The expression of *Fgf4* and *Fgf8* was completely inhibited in *Fgf16* knockdown pectoral fin buds at 42 hpf (Fig. 3Cj and k). *shh* expressed in the zone of polarizing activity (ZPA) is crucial for the formation of pectoral fin buds [18]. We also examined the expression of *shh*. The expression of *shh* was completely inhibited in *Fgf16* knockdown pectoral fin buds at 42 hpf (Fig. 3Cl).

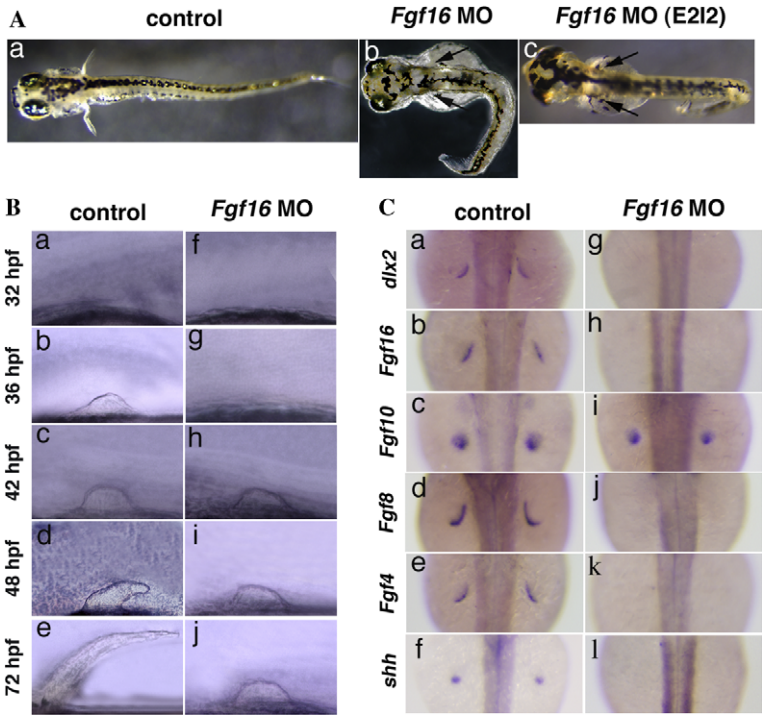

Fig. 3. Morphology and gene expression in pectoral fin buds of *Fgf16*-knockdown embryos. (A) Embryos were injected with 5-miss *Fgf16* MO (control) (a), *Fgf16* MO (b) or *Fgf16* MO (E2I2) (c) (~5.0 ng). Control MO-injected embryos at 5 hpf developed normally. In contrast, *Fgf16* MO or *Fgf16* MO (E2I2)-injected embryos showed bent trunks and no pectoral fin (arrows). (B) Embryos were injected with control MO (a–e) or *Fgf16* MO (f–j) (~5.0 ng). In control MO-injected embryos, the bud was a shallow dome at 36 hpf and further grew. At 48 hpf, the bud curved posteriorly and tapered. In contrast, no bud was observed at 32 and 36 hpf in the *Fgf16* MO-injected embryos. At 42 hpf, the bud was observed as a shallow dome. Even at 48 and 72 hpf, the buds remained shallow domes. (C) The expression of *dlx2*, *Fgf16*, *Fgf10*, *Fgf8*, *Fgf4*, and *shh* in fin buds of control MO-injected (a–f) and *Fgf16* MO-injected embryos (g–l) at 42 hpf was detected by whole mount in situ hybridization.

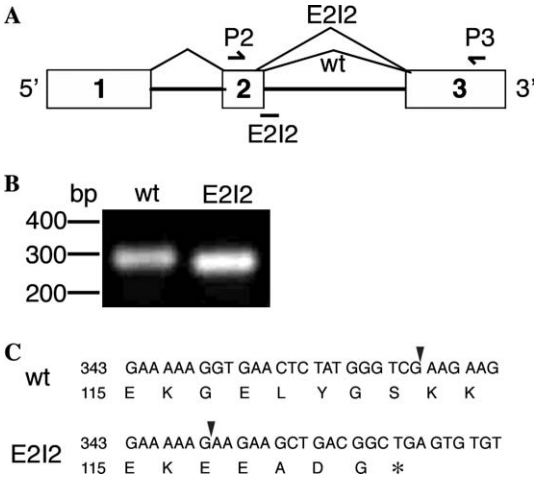

Fig. 4. Alteration of splicing by splice-site-targeted *Fgf16* MO (E2I2). (A) The coding region of Fgf16 was divided by two introns. Three blank boxes and two solid lines indicate three exons and two introns, respectively. The splice-site target is shown as E2I2. P2 and P3 indicate sites of P2 and P3 RT-PCR primers. (B) *Fgf16* cDNAs from wild-type and *Fgf16* MO (E2I2) were amplified by RT-PCR using P2 and P3 primers. The cDNAs were analyzed by 1.5% agarose gel eletrophoresis. After electrophoresis, the gel was stained with ethidium bromide. bp, base pair. (C) The nucleotide sequences of *Fgf16* cDNAs described above were determined. The numbers of the nucleotide sequence of the coding region and the amino acid sequence are shown. Arrowheads indicate splice-sites between exons two and three.

Expression of Fgf16, Fgf10, Fgf8, Fgf4, and shh in pectoral fin buds at different developmental stages

We examined the expression of *Fgf16* in pectoral fin buds at different developmental stages (28 – 36 hpf) (Fig. 5Ba–e). *Fgf16* was not detected in pectoral fin buds until 30 hpf. *FGF16* was first clearly detected in the AER at 34 hpf. We also examined the expression of *Fgf10*, *Fgf8*, *Fgf4*, and *shh* (Fig. 5Bf–y). The expression of *Fgf10* and *shh* was detected in the mesenchyme and the ZPA at all stages examined, respectively. In contrast, the expression of *Fgf8* and *Fgf4* was first detected in the AER at 36 hpf.

Expression of Fgf16 in pectoral fin buds of Fgf10, Fgf8, and Fgf4 knockdown embryos

To examine the relationship of Fgf16 signaling with Fgf10, Fgf8, and Fgf4 signaling in pectoral fin buds, we examined the expression of *Fgf16* in pectoral fin buds of *Fgf10*, *Fgf8*, and *Fgf4* MO-injected embryos at 36 hpf. The expression of *Fgf16* was completely inhibited in *Fgf10*-knockdown pectoral fin buds (Fig. 5Cb). In contrast, the expression of *Fgf16* was not inhibited in *Fgf4* and *Fgf8* knockdown pectoral fin buds (Fig. 5Cc and d).

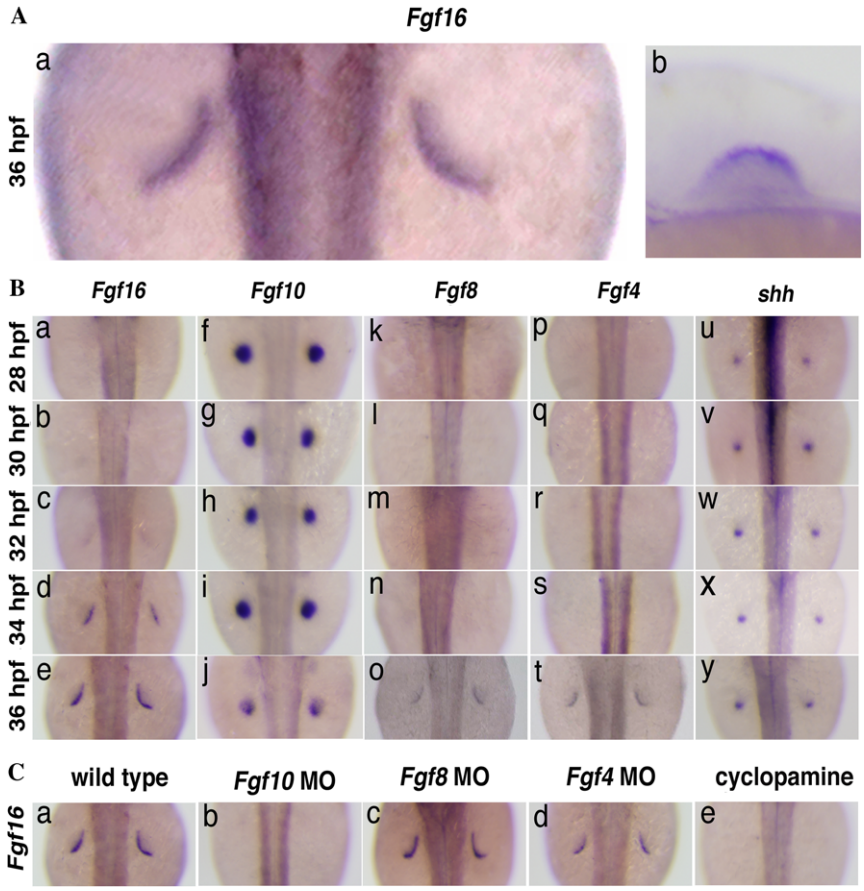

Fig. 5. Expression patterns of *Fgf16*, *Fgf10*, *Fgf8*, *Fgf4*, and *shh* in pectoral fin buds at different developmental stages and *Fgf16* expression in pectoral fin buds of gene knockdown embryos. (A) The expression of *Fgf16* in pectoral fin buds at 36 hpf was examined by whole mount in situ hybridization. Dorsal (a) and lateral (b) views of *Fgf16* expression ere shown. *Fgf16* was preferentially expressed in the AER. (B) The expression patterns of *Fgf16* (a–e), *Fgf10* (f–j), *Fgf8* (k–o), *Fgf4* (p–t), and *shh* (u–y) in pectoral fin buds at different developmental stages (28–36 hpf) were examined by whole mount in situ hybridization. (C) The expression of *Fgf16* in pectoral fin buds of wild-type (a), and *Fgf10* (b), *Fgf8* (c), and *Fgf4* (d) knockdown embryos at 36 hpf was examined by whole mount in situ hybridization. Cyclopamine completely blocked Hh signaling at the level of Smoothened that transduced Hh signaling. The expression of *Fgf16* in pectoral fin buds of cyclopamine-treated embryos at 36 hpf (e) was examined by whole mount in situ hybridization.

Expression of Fgf16 in pectoral fin buds of embryos in which shh signaling was inhibited by cyclopamine

Cyclopamine completely blocked Hh signaling at the level of Smoothened that transduced hedgehog signaling [27]. In pectoral fin buds, cyclopamine generates a phenocopy of the syu null mutant that disrupts *shh* [26]. We examined the expression of *Fgf16* and *Fgf10* as a control in cyclopamine-treated pectoral fin buds. The expression of *Fgf10* was not inhibited in cyclopamine-treated pectoral fin buds (data not shown). In contrast, the expression of *Fgf16* was completely inhibited in cyclopamine-treated pectoral fin buds at 36 hpf (Fig. 5Be).

Cell proliferation in fin buds

To identify proliferating cells in fin buds, phosphohistone H3 (H3P) was detected by immunohistochemistry using anti-H3P antibody. H3P-positive cells were detected in the mesenchyme of fin buds of control MO-injected embryos at 42 hpf (Fig. 6A and B). In contrast, the number of H3P-positive cells in the mesenchyme of fin buds of the *Fgf16* MO-injected embryos was greatly decreased (Fig. 6A and B). These results indicate that cell proliferation in the mesenchyme of the *Fgf16*-knockdown fin buds was greatly decreased.

A possible role of Fgf16 in pectoral fin bud formation

Fgf16 is a newly identified AER factor that plays crucial roles in zebrafish pectoral fin outgrowth. Fgf16 is required for cell proliferation and differentiation in the mesenchyme and AER of fin buds, respectively. Fgf10 in the mesenchyme is essential for the induction of *Fgf16* expression in the AER, although Fgf16 is not essential for the induction of *Fgf10* expression. In contrast, Fgf16 is essential for the induction of *Fgf4* and *Fgf8* expression in the AER, although Fgf4 and Fgf8 are not essential for *Fgf16* expression. The expression of *Fgf16* followed that of *Fgf10*, but preceded that of *Fgf4* and *Fgf8* during pectoral fin development. *shh* in the ZPA is essential for the induction of *Fgf16* expression. In addition, Fgf16 is also essential for *shh*

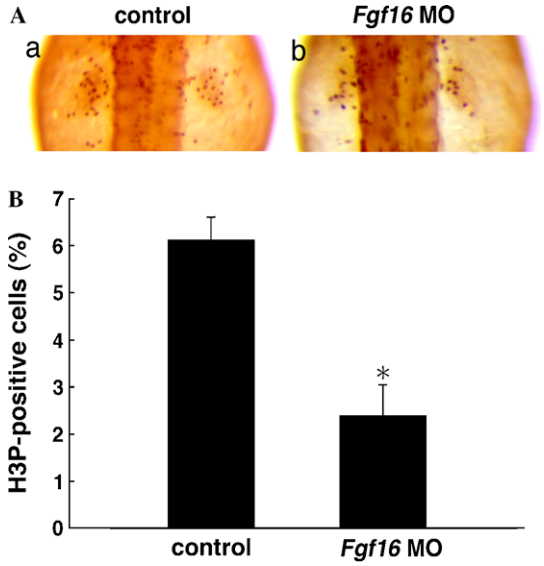

Fig. 6. Proliferation in fin buds. Proliferation was examined in fin buds of control MO or *Fgf16* MO-injected embryos at 42 hpf by immunohistochemistry using anti-H3P antibody. H3P-positive cells were detected in the mesenchyme of fin buds (A). For cell counts, sections of stained embryos were prepared. The rates of H3P-positive cells in the mesenchyme were quantitatively examined by counting H3P-positive and negative cells. The results are means ± SD of six independent sections from two embryos (B). An asterisk indicates a significant difference with the control ($P < 0.05$).

expression. These results indicate that Fgf16 functions downstream of Fgf10, a mesenchymal factor, signaling to induce the expression of *Fgf4* and *Fgf8* in the AER and that *Fgf16* in the AER and *shh* in the ZPA interact to induce and/or maintain each other's expression. These findings have revealed that Fgf16 plays a crucial role in pectoral fin bud outgrowth by mediating the interactions of AER-mesenchyme and AER-ZPA.

Acknowledgments

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