Available online at www.sciencedirect.com

ScienceDirect

Developmental Biology 303 (2007) 527–535

DEVELOPMENTAL BIOLOGY

www.elsevier.com/locate/ydbio

fibin, a novel secreted lateral plate mesoderm signal, is essential for pectoral fin bud initiation in zebrafish

Takashi Wakahara, Naoki Kusu, Hajime Yamauchi, Ikuo Kimura, Morichika Konishi, Ayumi Miyake, Nobuyuki Itoh *

Department of Genetic Biochemistry, Kyoto University Graduate School of Pharmaceutical Sciences, Sakyo, Kyoto 606-8501, Japan

Received for publication 23 October 2006; revised 17 November 2006; accepted 20 November 2006
Available online 1 December 2006

Abstract

We identified a novel secreted protein, fibin, in zebrafish, mice and humans. We inhibited its function in zebrafish embryos by injecting antisense *fibin* morpholino oligonucleotides. A knockdown of fibin function in zebrafish resulted in no pectoral fin bud initiation and abolished the expression of *tbx5*, which is involved in the specification of pectoral fin identification. The lack of pectoral fins in *fibin*-knockdown embryos was partially rescued by injection of *fibin* RNA. *fibin* was expressed in the lateral plate mesoderm of the presumptive pectoral fin bud regions. Its expression region was adjacent to that of *tbx5*. *fibin* expression temporally preceded *tbx5* expression in presumptive pectoral fin bud regions, and not abolished in *tbx5*-knockdown presumptive fin bud regions. In contrast, *fibin* expression was abolished in retinoic acid signaling-inhibited or *wnt2b*-knockdown presumptive fin bud regions. These results indicate that fibin is a secreted signal essential for pectoral fin bud initiation in that it potentially acts downstream of retinoic acid and wnt signaling and is essential for *tbx5* expression. The present findings have revealed a novel secreted lateral plate mesoderm signal essential for fin initiation in the lateral plate mesoderm.

Keywords: *fibin*; Fin; Limb; Lateral plate mesoderm; *tbx5*; Zebrafish

Introduction

Vertebrate limb/fin bud formation has been considered an excellent model for studying morphogenesis. Numerous factors have been implicated in the processes of limb identity and limb bud initiation and outgrowth in vertebrates (Martin, 1998). *T-box* (*Tbx*) genes encode a group of transcription factors with a highly conserved DNA-binding motif (T-box) (Muller and Herrmann, 1997). *Tbx* genes play key roles in the process of vertebrate limb/fin identity (Ahn et al., 2002; Ng et al., 2002; Takeuchi et al., 2003). Among *Tbx* genes, *Tbx4* is expressed in the developing hindlimb bud, whereas *Tbx5* is expressed in the forelimb bud. Tbx4 and Tbx5 are involved in the specification of hindlimb and forelimb identity, respectively. *Fgf10* is expressed in the limb bud-forming region of the lateral plate mesoderm (Ohuchi et al., 1997). *Fgf10* knockout mice have no limbs, indicating that Fgf10 is absolutely required for limb bud outgrowth (Min et al., 1998; Sekine et al., 1999). The molecular relationship between Fgf10 and Tbx5 during the initiation and specification of forelimb/pectoral fin identity has been revealed (Ng et al., 2002). Tbx5 is both necessary and sufficient for forelimb/pectoral fin bud initiation. Tbx5 acts to induce the expression of *Fgf10* in the signal cascade that directs forelimb/pectoral fin bud initiation. This cascade proceeds in the lateral plate mesoderm of presumptive forelimb/pectoral fin bud regions.

We identified Fibin, a novel secreted protein with no primary structural similarity, in humans, mice and zebrafish. Zebrafish *fibin* was expressed in the lateral plate mesoderm of presumptive pectoral fin bud regions. A knockdown of fibin function resulted in a complete lack of pectoral fin buds and abolished the expression of *Tbx5* in the lateral plate mesoderm. The present findings have indicated that fibin is a novel secreted

* Corresponding author. Fax: +81 75 753 4600.
E-mail address: itohnobu@pharm.kyoto-u.ac.jp (N. Itoh).

0012-1606/$ - see front matter © 2006 Elsevier Inc. All rights reserved.
doi:10.1016/j.ydbio.2006.11.041

lateral plate mesoderm signal essential for the expression of *tbx5* during pectoral fin bud initiation.

Materials and methods

Fish maintenance

Zebrafish (*Danio rerio*) were maintained as described (Westerfield, 2000). Embryos were obtained by natural spawning and cultured at 28.5 °C in Zebrafish Ringer's solution. The developmental stages of embryos were determined by the hours post fertilization (hpf) and by morphological features as described (Kimmel et al., 1995).

Isolation of mouse, human and zebrafish Fibin cDNAs

Amino acid sequences predicted from mouse cDNAs of unknown function in GenBank were randomly analyzed using PSORT (Prediction of Protein Sorting Signals and Localization Sites in Amino Acid Sequences) (http://psort.ims.u-tokyo.ac.jp/form2.html). Many cDNAs encoding putative secreted proteins were identified. We isolated their full-length cDNAs by polymerase chain reaction (PCR) with the embryonic cDNA as a template. We termed one of them mouse *Fibin*.

Human and zebrafish *Fibin* cDNAs were also identified in a homology search of human and zebrafish genomic and cDNA sequences in GenBank with the amino acid sequence of mouse *Fibin*. The full-length cDNAs of human and zebrafish *Fibin* were isolated by PCR with human placenta and zebrafish embryo cDNA as templates, respectively.

Construction of a recombinant expression vector with mouse Fibin cDNA

The mouse *Fibin* cDNA with a DNA fragment of 75 base pairs encoding an E tag (GAPVPYPDPLEPR) and a His_6 tag (HHHHHH) at the 3′ terminus of the coding region was constructed in a expression vector DNA, *pcDNA3.1(+)* (Invitrogen).

Detection of recombinant mouse Fibin by Western blotting

COS-7 cells plated at ~90% confluency on 24-well plates were transfected with the expression vector using a transfection kit, Lipofectamine 2000 (Invitrogen) and were cultured at 37 °C for 72 h in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (JRH Biosciences). The culture medium and cell lysate of COS-7 cells transfected with the expression vector were separated by SDS–polyacrylamide gel (12.5%) electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane (Hybond-ECL, Amersham Biosciences). The protein with the E tag on the membrane was detected using anti-E tag antibody (Amersham Biosciences) as described (Miyake et al., 1998).

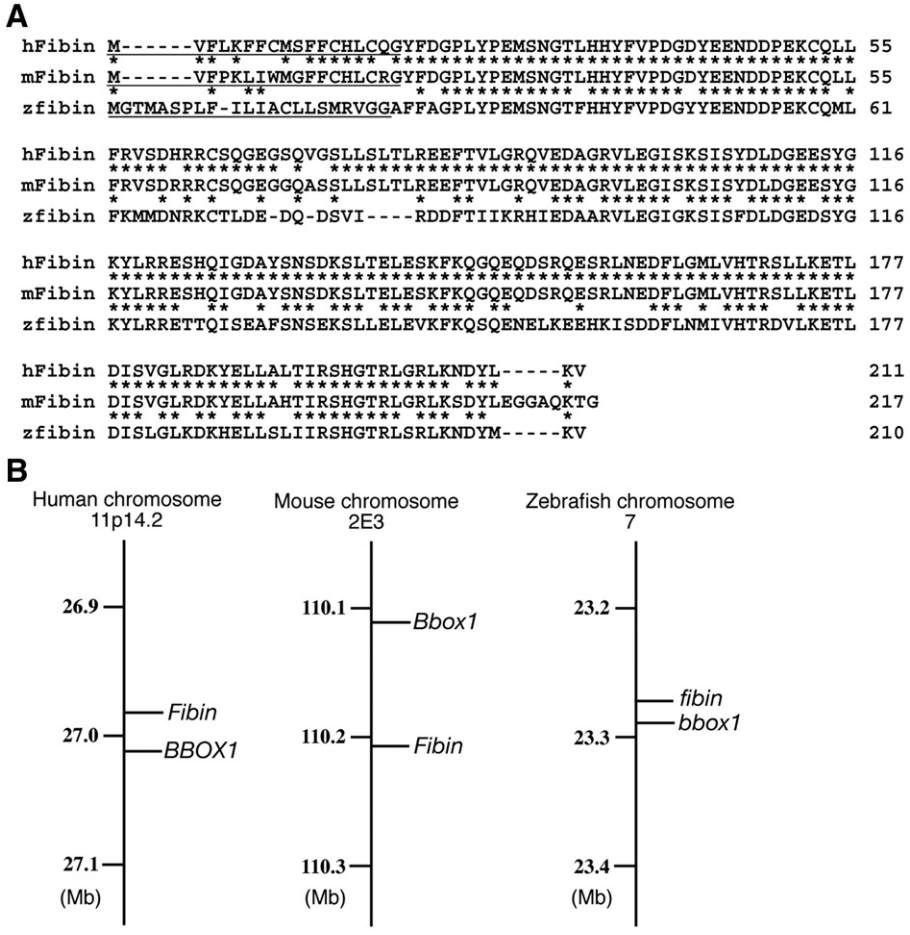

Fig. 1. Molecular analysis of *Fibin*. (A) Comparison of amino acid sequences of human, mouse and zebrafish Fibin. The numbers refer to amino acid positions of human, mouse and zebrafish Fibin. Asterisks indicate identical amino acid residues of the sequences. Underlined amino acids indicate putative secreted signal sequences. Dashes indicate introduced gaps. (B) Syntenic relationship between human chromosome 11p14.2, mouse chromosome 2E3 and zebrafish chromosome 7. Human, mouse and zebrafish *Fibin* genes are closely linked to human, mouse and zebrafish *Bbox1* genes, respectively. Mb, megabase.

Detection of recombinant mouse Fibin by immunohistochemistry

COS-7 cells plated at ~90% confluency on 24-well plates were transfected with the expression vector as described above and cultured at 37 °C for 48 h in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (JRH Biosciences). The transfected COS-7 cells (~1×10^3 cells/cm^2) were further cultured at 37 °C for 24 h on poly-L-lysine-coated cover glasses (24×40 mm) in 60 mm dishes and stained with a standard protocol using antibodies described below. To detect Fibin, mouse anti-E tag antibody (Pharmacia Biotech) (1:500 dilution) and FITC-conjugated goat anti-mouse IgG (Sigma) (1:500) were used as primary and secondary antibodies, respectively. To detect mannosidase II, a marker protein for the Golgi apparatus (Moremen and Touster, 1986), rabbit anti-mannosidase II antibody (Abcam) (1:200) and TRITC-conjugated goat anti-rabbit antibody (Sigma) (1:400) were used as primary and secondary antibodies, respectively.

Whole mount in situ hybridization

Digoxigenin-labeled RNA probes were synthesized by in vitro transcription using T7 or SP6 RNA polymerase. Zebrafish *fibin* and mouse *Fibin* probes were synthesized using full-length cDNA-containing plasmids. Another probe used was zebrafish *tbx5* (Tamura et al., 1999). Whole mount in situ hybridization was performed using zebrafish or mouse embryos as described (Koshida et al., 1998; Laurikkala et al., 2003). Transverse sections (16 μm) were prepared from zebrafish embryos embedded in agar using a cryostat as described (http://zfin.org/zf_info/zfbook/chapter8/8.2.html).

Gene knockdown by morpholinos

Morpholino oligonucleotides (MOs) were synthesized by Gene-Tools, LLC (Corvallis, OR). The sequences of MOs used are as follows: *fibin* MO, 5′–GGAAGCCATCGTACCCATCTCCCTT–3′; five-base mismatched *fibin* (control) MO, 5′–GcAAcCCATCGTAgCCATCaCCgTT–3′; *fibin* MO2, 5′–ACAGAGGGGAAGCCATCGTACCCAT–3′; *tbx5* MO, 5′–GGTGTC-TTCACTGTCCGCCATGTCG–3′; *wnt2b* MO, 5′–ACCCAACTCCATCACA-CTCTGGCAT–3′. Lower case letters in control *fibin* MO indicate mismatches with nucleotides in *fibin* MO. MOs were diluted in Danieau buffer (Nasevicius and Ekker, 2000). *fibin* MO (6.7 μg/μl) and control *fibin* MO (6.7 μg/μl) were injected at a volume of 0.5 nl into two-cell embryos. *fibin* MO2 (13 μg/μl) was injected at a volume of 0.3 nl into two-cell embryos. *tbx5* MO (1.3 μg/μl) was injected at a volume of 0.6 nl into two-cell embryos. *wnt2b* MO (13 μg/μl) was injected at a volume of 0.75 nl into two-cell embryos.

fibin morpholino rescue experiments with fibin RNA

Capped zebrafish *fibin* sense RNA was synthesized using the mMESSAGE mMACHINE kit (Ambion) according to the manufacturer's protocol from a linearized *pCS2+* plasmid containing the entire coding region of zebrafish *fibin* cDNA. For *fibin* morpholino rescue experiments, 100 pg of *fibin* sense RNA was injected separately, immediately following the injection of *fibin* MO into two-cell embryos.

DEAB treatment

Zebrafish embryos at 5 hpf were treated with 4-diethylaminobenzaldehyde (DEAB) (10 μM) for 9 or 11 h. The expression of *fibin* was examined in DEAB-treated embryos at 14 or 18 hpf by whole mount in situ hybridization.

Results

Identification of Fibin in mice and humans

We identified a mouse embryonic cDNA encoding a novel putative secreted protein by analyzing amino acid sequences from mouse cDNAs of unknown function in the GenBank nucleotide sequence database with a computer program for the prediction of protein localization sites in cells (PSORT). The full-length cDNA was isolated by PCR with mouse embryonic cDNA (E14.5) as a template. The cDNA encoded a protein of 217 amino acids (Accession Number in GenBank/EBML/DDBJ nucleotide sequence databases: AB236893) (Fig. 1A). We termed it mouse Fibin (fin bud initiation factor), because it played a crucial role in zebrafish pectoral fin bud initiation as described below. Fibin is a unique protein with no primary structural similarity. We also identified the human *Fibin* gene by BLAST-searching human genomic sequences with the amino acid sequence of mouse Fibin. The full-length cDNA of human *Fibin* was isolated from human placental cDNA.

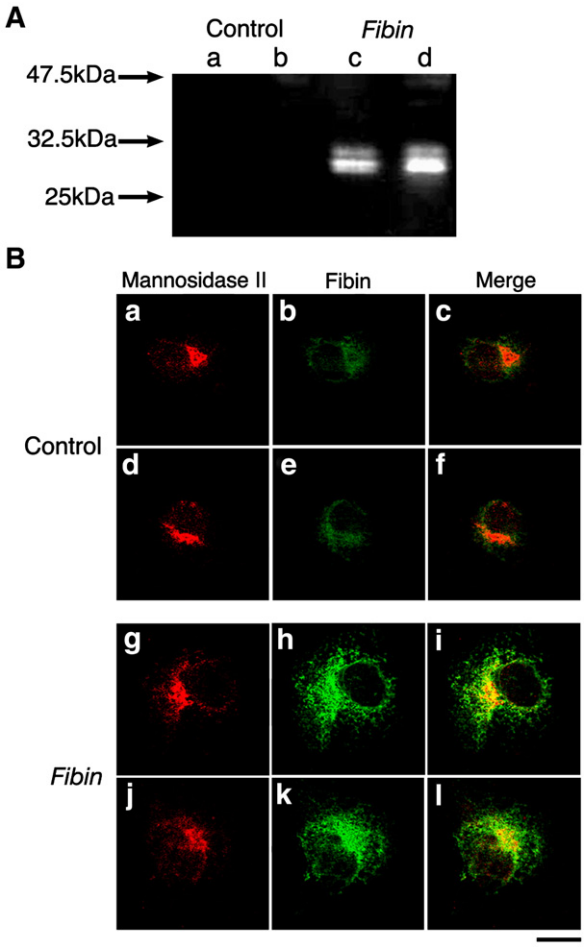

Fig. 2. Detection of recombinant mouse Fibin expressed in COS-7 cells. (A) COS-7 cells were transfected with the empty vector (Control) or the mouse *Fibin*-expression vector (*Fibin*). The cell lysate (a, c) and culture medium (b, d) of the transfected COS-7 cells were separated by SDS–polyacrylamide gel electrophoresis under reducing conditions and transferred onto a nitrocellulose membrane. The protein with the E tag on the membrane was detected using anti-E tag antibody. (B) COS-7 cells transfected with the empty vector (Control) (a–f) or the mouse *Fibin*-expression vector (*Fibin*) (g–l) were examined by immunohistochemistry using anti-mannosidase II antibody for the Golgi apparatus (a, d, g, j) and anti-E tag antibody for recombinant Fibin (b, e, h, k). The signals obtained by immunohistochemistry using anti-mannosidase II antibody and anti-E tag antibody were merged (c, f, i, l). Scale bar=20 μm.

Human Fibin of 211 amino acids (Accession Number: AB236892) was highly similar (~94% amino acid identity) to mouse Fibin (Fig. 1A). The coding region of *Fibin* is coded by a single exon (data not shown). Mouse *Fibin* is closely linked to the γ-butyrobetaine hydroxylase gene (*Bbox1*) on chromosome 2 at E3. Human *Fibin* is also closely linked to *BBOX1* on chromosome 11 at p14.2 (Fig. 1B).

Fibin has a putative secreted signal sequence at its amino-terminus (Fig. 1A) but no typical transmembrane sequence, nuclear localization signal sequence or endoplasmic reticulum retention signal sequence according to an analysis with PSORT. Fibin was expected to be a secreted protein. To examine whether Fibin is a secreted protein, we expressed mouse cDNA encoding Fibin with an E-tag in cultured cells, COS-7 cells. Both the culture medium and cell lysate were examined by Western blotting with anti-E tag antibody. A major band of ~28 kDa was detected in the culture medium (Fig. 2A, d). The observed molecular mass was slightly larger than the calculated molecular mass of recombinant Fibin protein (25, 302 Da). The band was also detected in the cell lysate (Fig. 2A, c). We also examined the cellular localization of Fibin in the cells by immunohistochemical analysis with anti-E tag antibody. Fibin was mainly co-localized with mannosidase II, a marker protein for the Golgi apparatus (Moremen and Touster, 1986), indicating that most of the Fibin in the cells was mainly present on the Golgi apparatus (Fig. 2B).

Identification and expression of fibin in zebrafish

The zebrafish is a useful model for elucidation of the functions of genes in vertebrates in vivo. Zebrafish orthologs exist for most mammalian genes, and zebrafish organs are also functionally and morphologically similar to mammalian organs (Shin and Fishman, 2002). In addition, the functions of zebrafish genes can be easily and effectively blocked by antisense morpholino oligonucleotides (MO) in vivo (Nasevicius and Ekker, 2000). We identified two zebrafish *fibin*-like genes by BLAST-searching the nucleotide sequence database with the amino acid sequences of mouse Fibin. The proteins encoded by them were significantly similar (~58% and ~51% amino acid identities) to mouse Fibin. As one of them encoding the protein with ~58% amino acid identity is closely linked to *bbox1* on chromosome 7 (Fig. 1B). We expect that the gene is zebrafish *fibin*, although the chromosomal localization of the other remains to be determined. We isolated a full-length cDNA of zebrafish *fibin* from the embryo cDNA (Accession Number: AB236894). Zebrafish fibin of 210 amino acids also has a putative secreted signal sequence at its amino-terminus (Fig. 1A) but no typical transmembrane sequence, nuclear localization signal sequence or endoplasmic reticulum retention signal sequence. The coding region of *fibin* is also coded by a single exon (data not shown). *Fibin* was also identified in several vertebrates

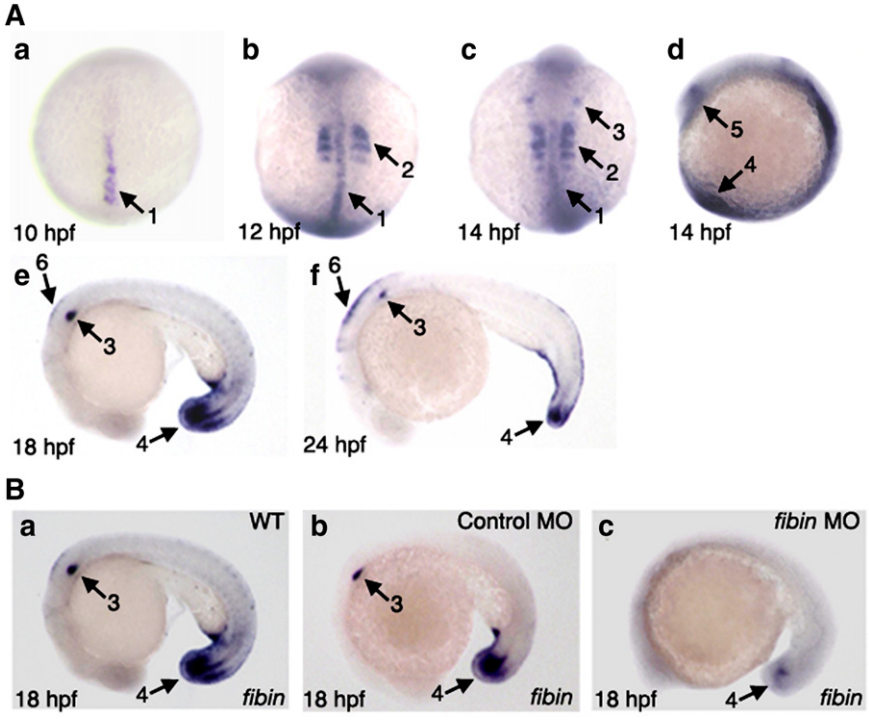

Fig. 3. Expression of *fibin* in zebrafish embryos. (A) The spatiotemporal expression pattern of *fibin* in zebrafish embryos (10 ~ 24 hpf) was examined by whole mount in situ hybridization. *fibin* was expressed in the notochord at 10 hpf (a) and in the notochord and somites at 12 and 14 hpf (b, c). *fibin* was also expressed in the lateral plate mesoderm, tail buds and the forebrain at 14 hpf (c, d). At 18 and 24 hpf, *fibin* was predominantly expressed in tail buds and presumptive pectoral fin bud regions. In addition, fibin was also expressed in the alar plate (e, f). (B) The expression of *fibin* in *fibin* MO-injected zebrafish embryos. The expression of *fibin* in wild-type (a), antisense five-base mismatched *fibin* (Control) MO- (b) and antisense *fibin* MO- (c) injected embryos at 18 hpf was examined by whole mount in situ hybridization. 1, notochord; 2, somites; 3, lateral plate mesoderm or presumptive pectoral fin bud regions; 4, tail; 5, forebrain; 6, alar plate of the hindbrain.

including rats, cattle, chimpanzees, orangutans, dogs, chickens and pufferfish by BLAST-searching GenBank/EBML/DDBJ nucleotide sequence databases with the amino acid sequence of mouse Fibin. However, *Fibin* was not identified in invertebrates (data not shown). We examined the spatiotemporal expression pattern of *fibin* in zebrafish embryos during 5~24 hpf by whole mount in situ hybridization (Fig. 3A). The expression of *fibin* was not detected in embryos during 5~9 hpf (data not shown). The expression of *fibin* was first detected in the notochord at 10 hpf. At 12 and 14 hpf (the 6- and 10-somite stages), *fibin* was expressed in the notochord, somites and tail. At 14 hpf, *fibin* was also expressed in the forebrain and the lateral plate mesoderm anterior to somite 1. At 18 and 24 hpf, *fibin* was predominantly expressed in the tail, presumptive pectoral fin bud regions and the alar plate of the hindbrain.

Inhibition of fibin function results in defects in formation of pectoral fin buds in zebrafish

To examine the role of *fibin* in zebrafish development, we injected antisense *fibin* and five-base mismatched *fibin* (control) MOs into zebrafish 2-cell embryos. The *fibin* MO sequence (25 nucleotides) is complementary to the nucleotide sequence including 18 nucleotides of the coding region and 7 nucleotides of the 5′ non-coding region. The expression of *fibin* was examined in *fibin* MO-injected embryos. The expression of *fibin* was greatly decreased in the tail and the presumptive pectoral fin bud region of *fibin*-knockdown embryos at 18 hpf, although the expression was not affected in control MO-injected embryos (Fig. 3B).

The morphology of the embryos was observed at 36 hpf, 48 hpf and 4 days post-fertilization (dpf). The *fibin* MO-injected

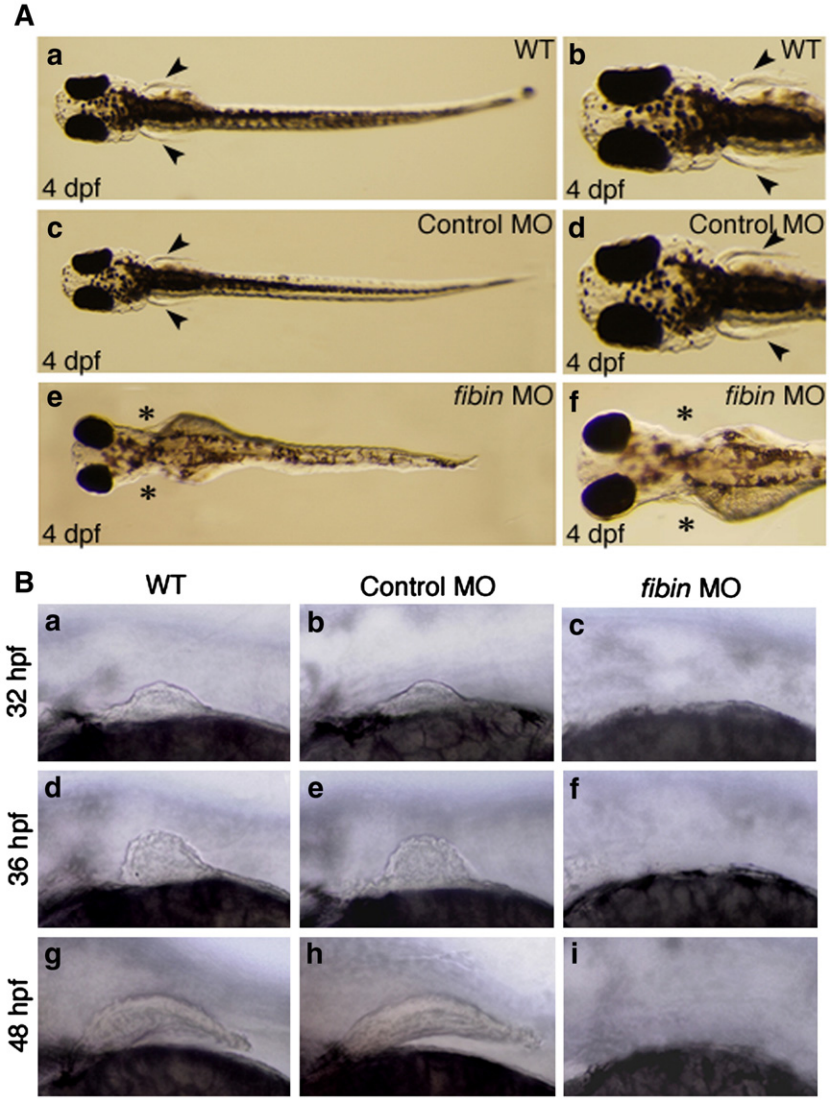

Fig. 4. Morphology of pectoral fin buds of *fibin* knockdown embryos. (A) Embryos were injected with antisense five-base mismatched *fibin* MO (Control) and *fibin* MO. The morphology in wild-type (a, b), control (c, d) and *fibin* knockdown (e, f) embryos was observed at 4 dpf. Arrowheads and asterisks indicate pectoral fins and no pectoral fins, respectively. (B) The morphology of pectoral fin buds was observed in wild-type (a, d, g), control (b, e, h) and *fibin* knockdown (c, f, i) embryos at 32~48 hpf.

embryos had no pectoral fins (Figs. 4A, B and Table 1). In contrast, the control MO-injected embryos developed normally (Figs. 4A, B and Table 1). We also examined the pectoral fin bud formation in the *fibin* MO-injected embryos early in development (32∼48 hpf). In contrast to the wild-type and control MO-injected embryos, no pectoral fin bud initiation was observed in the *fibin* MO-injected embryos (Fig. 4B). We also injected antisense *fibin* MO2 into zebrafish 2-cell embryos. The *fibin* MO2 sequence (25 nucleotides) is complementary to the nucleotide sequence including the initiation codon but not the 5′ non-coding region. The phenotype of *fibin* MO2-injected embryos was similar to that of *fibin* MO-injected embryos. The *fibin* MO2-injected embryos at 36 hpf, 48 hpf and 4 dpf also had no pectoral fins (Table 1).

We prepared capped zebrafish *fibin* RNA including the entire coding region but not the 5′ non-coding region. As the *fibin* MO sequence includes 7 nucleotides of the 5′ non-coding region, the translation of *fibin* RNA should be unaffected by the *fibin* MO. We examined whether *fibin* RNA could rescue the phenotype of *fibin* MO embryos at 4 dpf. Injection of *fibin* RNA (100 pg/embryo) into wild-type embryos did not affect the phenotype (Table 2). In contrast, the lack of pectoral fins in *fibin* MO embryos was partially rescued by injection of *fibin* RNA (Table 2). These results indicate that fibin is essential for the initiation of pectoral fin bud formation.

fibin is required for tbx5 expression in the lateral plate mesoderm of presumptive pectoral fin bud regions

tbx5 is expressed in presumptive pectoral fin bud regions and is involved in the specification of pectoral fin identity in zebrafish. tbx5 is therefore essential for fin bud formation in zebrafish (Ng et al., 2002). The expression of *tbx5* was observed at the lateral plate mesoderm of presumptive pectoral fin bud regions at 18 and 26 hpf (Figs. 5a and 6c). However, the expression of *tbx5* was abolished or decreased in the lateral plate mesoderm of *fibin*-knockdown embryos at 18 hpf ($n=57$, abolished expression 8/57, decreased expression 21/57) (data not shown) and 26 hpf ($n=48$, abolished expression 15/48, decreased expression 18/48) (Fig. 5b). In contrast, the expression of *fibin* was unchanged or increased in the presumptive pectoral fin bud regions of *tbx5*-knockdown embryos at 18 hpf ($n=44$, unchanged expression 14/44, increased expression 25/44) (Figs. 5c, d).

Table 1
Phenotypes resulting from injection of *fibin* MOs into zebrafish embryos

		Number of observed phenotypes			Total number of embryos
		Normal fin	Partial fin	No fin	
fibin MO	(3.5 ng)	32	16	31	79
fibin MO2	(4.0 ng)	13	29	31	73
Control MO	(3.5 ng)	39	0	0	39

Antisense *fibin* MOs (MO and MO2) and five-base mismatched *fibin* (control) MO were injected into zebrafish 2-cell embryos. The morphology of the embryos was observed at 36 hpf, 48 hpf and 4 dpf.

Table 2
Phenotypes resulting from injection of *fibin* RNA and *fibin* MO into zebrafish embryos

		Number of observed phenotypes			Total number of embryos
		Normal fin (% of total)	Partial fin (% of total)	No fin (% of total)	
fibin RNA	(100 pg)	32 (100)	0 (0)	0 (0)	32
fibin MO	(3.5 ng)	11 (29.7)	8 (21.6)	18 (48.6)	37
fibin MO+*fibin* RNA		22 (44.0)	13 (26.0)	15 (30.0)	55

fibin RNA and/or *fibin* MO were injected into zebrafish 2-cell embryos. The morphology of the embryos was observed at 4 dpf.

wnt2b and raldh2 are required for fibin expression in the lateral plate mesoderm of presumptive pectoral fin bud regions

wnt2b was reported to be expressed in the intermediate mesoderm of presumptive pectoral fin bud regions. tbx5 acts downstream of wnt2b to induce fin bud formation (Ng et al., 2002). We examined the expression of *fibin* in the lateral plate mesoderm and presumptive pectoral fin bud regions of *wnt2b*-knockdown embryos at 14 and 18 hpf by injection of *wnt2b* MO, respectively. The expression of *fibin* was abolished or decreased in *wnt2b*-knockdown lateral plate mesoderm and presumptive pectoral fin bud regions at 14 hpf ($n=37$, abolished expression 16/37, decreased expression 7/37) (data not shown) and at 18 hpf ($n=50$, abolished expression 16/50, decreased expression 22/50) (Fig. 5e), respectively. Retinaldehyde dehydrogenase type 2 (raldh2) is an enzyme involved in retinoic acid biosynthesis. The

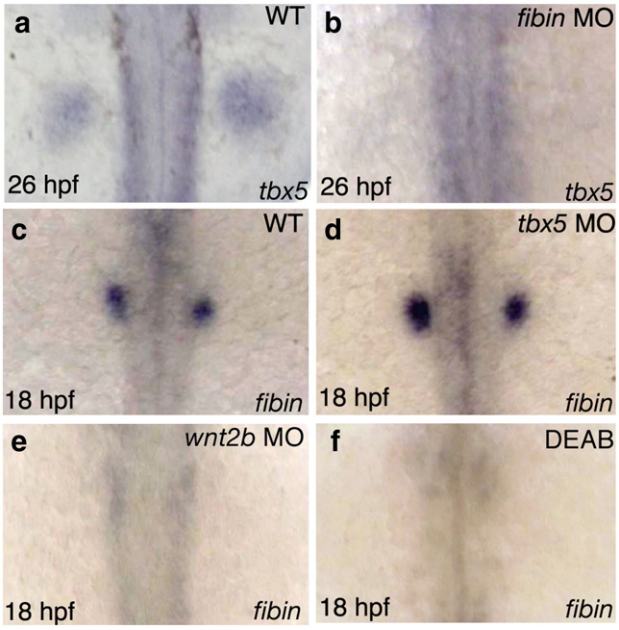

Fig. 5. Expression of *tbx5* and *fibin* in presumptive pectoral fin bud regions of gene knockdown or DEAB-treated embryos. The expression of *tbx5* was examined in wild-type (a) and *fibin*-knockdown (b) presumptive pectoral fin bud regions at 26 hpf by whole mount in situ hybridization. The expression of *fibin* was examined in wild-type (c), *tbx5*-knockdown (d), *wnt2b*-knockdown (e) and DEAB-treated (f) presumptive pectoral fin bud regions at 18 hpf.

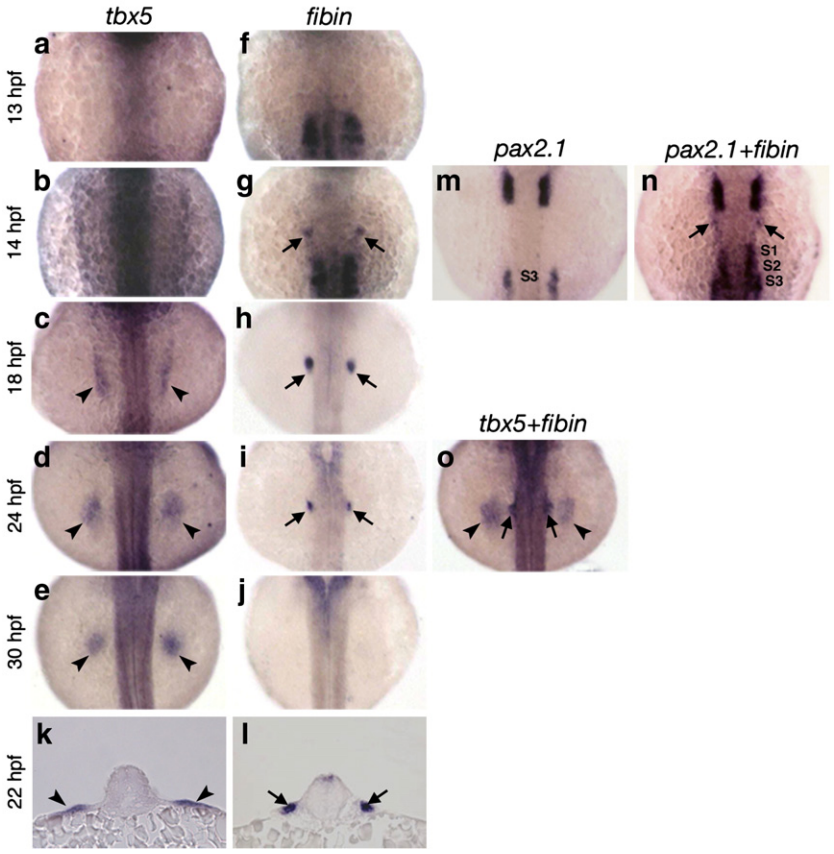

Fig. 6. Expression of *tbx5* and *fibin* in pectoral fin buds during zebrafish embryonic development. The expression of *tbx5* (a–e) and *fibin* (f–j) was examined in presumptive pectoral fin bud regions during different developmental stages (13~30 hpf) by whole mount in situ hybridization. The expression of *tbx5* and *fibin* in presumptive pectoral fin bud regions at 22 hpf was also examined at transverse sections (16 μm) (k, l). The expression of *pax2.1* was examined in embryos at 14 hpf by whole mount in situ hybridization (m). The expression of *pax2.1* and *fibin* was examined in embryos at 14 hpf by double whole mount in situ hybridization (n). The expression of *tbx5* and *fibin* was also examined in presumptive pectoral fin bud regions at 24 hpf by double whole mount in situ hybridization (o). Arrowheads and arrows indicate the expression of *tbx5* and *fibin* in presumptive pectoral fin bud regions, respectively. S1, S2 and S3 indicate the locations of somite 1, somite 2 and somite 3, respectively.

neckless/*raldh2*-mutant (*nls*) zebrafish lacks pectoral fins. *raldh2* is the earliest gene known to be required for pectoral fin bud induction (Gibert et al., 2006). We examined the expression of *fibin* in presumptive pectoral fin bud regions of zebrafish embryos at 14 and 18 hpf treated with DEAB, a competitive, reversible inhibitor of retinaldehyde dehydrogenases (Perz-Edwards et al., 2001). The expression of *fibin* was abolished in DEAB-treated lateral plate mesoderm and presumptive pectoral fin bud regions at 14 hpf (n=58, 58/58) (data not shown) and 18 hpf (n=47, 47/47) (Fig. 5f), respectively.

Expression of tbx5 and fibin in presumptive fin bud regions at different developmental stages

We examined the expression of *tbx5* in presumptive fin bud regions at different developmental stages (13~30 hpf) by whole mount in situ hybridization (Figs. 6a–e). At 14 hpf (the 10-somite stage), only the blurry expression of *tbx5* in the lateral plate mesoderm was observed. *tbx5* expression was first clearly observed in presumptive pectoral fin bud regions of the lateral plate mesoderm at 18 hpf, and increased gradually until 30 hpf.

We also examined the expression of *fibin* in presumptive fin bud regions (Figs. 6f–j). In contrast to *tbx5*, the expression of *fibin* began at an earlier stage in the lateral plate mesoderm. At 14 hpf, *fibin* was first clearly detected in the lateral plate mesoderm anterior to somites where *fibin* was also expressed. *fibin* was abundantly expressed in the lateral plate mesoderm at 18 hpf. However, the expression of *fibin* gradually decreased from 24 hpf, and was no longer detectable at 30 hpf.

pax2.1 was expressed in the lateral mesoderm located lateral to somite 3 (Fig. 6m) (Begemann et al., 2001). To identify the location of *fibin* expression in the lateral plate mesoderm, we examined the expression of *fibin* and *pax2.1* at 14 hpf by double whole mount in situ hybridization (Fig. 6n). *fibin* was expressed in somites 1–4 and the lateral plate mesoderm anterior to somite 1.

The location of *fibin* expression at 24 hpf appeared to be distinct from but adjacent to that of *tbx5* expression (Figs. 6d, i). We also examined the expression of *fibin* and *tbx5* at 24 hpf by double whole mount in situ hybridization. The location of *fibin* expression was clearly distinct from but adjacent to that of *tbx5* expression (Fig. 6o). We also examined the expression of *tbx5*

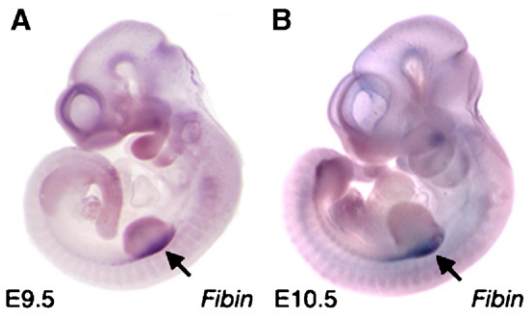

Fig. 7. Expression of *Fibin* in mouse embryos. The expression of *Fibin* was examined in mouse embryos at E9.5 (A) and E10.5 (B) by whole mount in situ hybridization. Arrows indicate the expression of *Fibin* in the forelimb bud regions.

and *fibin* in transverse sections at 22 hpf. *fibin* was expressed in the inner region of the lateral plate mesoderm distinct to the location of *tbx5* expression (Figs. 6k, l).

We also examined the expression of *Fibin* in mouse embryos at E9.5 and 10.5 by whole mount in situ hybridization (Fig. 7). *Fibin* was abundantly expressed in the forelimb buds. The regional expression profile of mouse *Fibin* in the forelimb buds is similar to that of zebrafish *fibin* in the pectoral fin buds.

Discussion

Fibin is a unique secreted protein

We identified a novel secreted protein, Fibin, having no primary structural similarity. The *Fibin* gene was identified in several vertebrates but not invertebrates, indicating that *Fibin* is a gene specific for vertebrates. Although Fibin is a secreted protein, it is also detected in the Golgi apparatus, which plays a role in the biosynthetic–secretory pathway where secreted proteins are transported from the endoplasmic reticulum to the secretory vesicle. Fibin is expected to be a secreted protein transported from the endoplasmic reticulum to the secretory vesicle.

fibin is essential for pectoral fin bud formation

Zebrafish *fibin* was expressed in the notochord, somites, the tail, the forebrain, the alar plate of the hindbrain and presumptive pectoral fin bud regions of the embryos. *fibin* MO decreased *fibin* mRNA levels in presumptive pectoral fin bud regions as well as the tail. The loss of *fibin* mRNA indicates that fibin might contribute to the autoregulation of its own gene expression. These results indicated that *fibin* MO inhibited the function of fibin. The most prominent phenotype of *fibin*-knockdown embryos was a complete lack of pectoral fins. Even at the early developmental stages, the most prominent phenotype of *fibin*-knockdown embryos was also a complete lack of fin buds. These results indicated that fibin is essential for the initiation of pectoral fin bud formation.

Possible mechanism of fibin action in pectoral fin bud formation

The expression of *tbx5* in presumptive pectoral fin bud regions was abolished in *fibin*-knockdown embryos. In contrast, the expression of *fibin* was not abolished in *tbx5*-knockdown presumptive pectoral fin bud regions. In addition, *fibin* expression temporally preceded *tbx5* expression in presumptive pectoral fin bud regions. These results clearly indicate that fibin is essential for pectoral fin bud formation in that it induces the expression of *tbx5*.

fibin and *tbx5* were expressed in different regions of the lateral plate mesoderm at presumptive fin bud regions. *tbx5* was expressed in the outer region of the lateral plate mesoderm. In contrast, *fibin* was expressed in the inner region of the lateral plate mesoderm. The location of *fibin* expression was adjacent to that of *tbx5* expression. As fibin is a secreted protein, fibin might be a permissive signal for the expression of *tbx5* in the adjacent region within the lateral plate mesoderm.

fgf and wnt signalings play crucial roles in fin bud formation. fgf24 acts downstream of tbx5 to activate the expression of *fgf10* that directs pectoral fin bud initiation (Fischer et al., 2003; Draper et al., 2003). fgf16 in the apical ectodermal ridge acts downstream of fgf10 to promote pectoral fin bud outgrowth (Nomura et al., 2006). In contrast, tbx5 acts downstream of wnt2b (Ng et al., 2002). The expression of *fibin* was completely abolished in *wnt2b*-knockdown presumptive pectoral fin bud regions. These results indicate that fibin functions downstream of wnt2b to induce the expression of *tbx5*. However, although *wnt2b* should be expressed in the intermediate mesoderm (Ng et al., 2002), we could not detect it in any presumptive pectoral fin bud regions of wild-type and *fibin*-knockdown embryos. *raldh2* is the earliest gene known to be required for pectoral fin bud induction (Gibert et al., 2006). We examined the expression of *fibin* in presumptive pectoral fin bud regions of zebrafish embryos treated with DEAB, an inhibitor of retinaldehyde dehydrogenases (Perz-Edwards et al., 2001). The expression of *fibin* was completely abolished in DEAB-treated presumptive pectoral fin bud regions, indicating that fibin functions downstream of raldh2.

In conclusion, fibin is a novel secreted protein identified in zebrafish, mice and humans. Zebrafish *fibin* was expressed in the lateral plate mesoderm of pectoral fin bud regions on early stage. fibin is essential for the initiation of pectoral fin bud initiation in that it potentially acts downstream of retinoic acid and wnt signaling and is essential for *tbx5* expression in the lateral plate mesoderm. In the lateral plate mesoderm, tbx5 acts to induce the expression of *fgf24* and *fgf10* in the signal cascade that directs pectoral fin bud initiation. The present findings have revealed a novel secreted lateral plate mesoderm signal essential for fin initiation in the lateral plate mesoderm.

Acknowledgments

We thank K. Vogeli and G. Martin for useful suggestions. This work was supported by the 21st Century COE Program from the Ministry of Education, Culture, Sports, Science and

Technology, Japan (T.W.), the Takeda Science Foundation, Japan (N.I.) and the Mitsubishi Foundation, Japan (N.I.).

References

Ahn, D.G., Kourakis, M.J., Rohde, L.A., Silver, L.M., Ho, R.K., 2002. T-box gene *tbx5* is essential for formation of the pectoral limb bud. Nature 417, 754–758.

Begemann, G., Schilling, T.F., Rauch, G.J., Geisler, R., Ingham, P.W., 2001. The zebrafish *neckless* mutation reveals a requirement for *raldh2* in mesodermal signals that pattern the hindbrain. Development 128, 3081–3094.

Draper, B.W., Stock, D.W., Kimmel, C.B., 2003. Zebrafish *fgf24* functions with *fgf8* to promote posterior mesodermal development. Development 130, 4639–4654.

Fischer, S., Draper, B.W., Neumann, C.J., 2003. The zebrafish *fgf24* mutant identifies an additional level of Fgf signaling involved in vertebrate forelimb initiation. Development 130, 3515–3524.

Gibert, Y., Gajewski, A., Meyer, A., Begemann, G., 2006. Induction and prepatterning of the zebrafish pectoral fin bud requires axial retinoic acid signaling. Development 133, 2649–2659.

Kimmel, C.B., Ballard, W.W., Kimmel, S.R., Ullmann, B., Schilling, T.F., 1995. Stages of embryonic development of the zebrafish. Dev. Dyn. 203, 253–310.

Koshida, S., Shinya, M., Mizuno, T., Kuroiwa, A., Takeda, H., 1998. Initial anteroposterior pattern of the zebrafish central nervous system is determined by differential competence of the epiblast. Development 125, 1957–1966.

Laurikkala, J., Kassai, Y., Pakkasjarvi, L., Thesleff, I., Itoh, N., 2003. Identification of a secreted BMP antagonist, ectodin, integrating BMP, FGF, and SHH signals from the tooth enamel knot. Dev. Biol. 264, 91–105.

Martin, G.R., 1998. The roles of FGFs in the early development of vertebrate limbs. Genes Dev. 12, 1571–1586.

Min, H., Danilenko, D.M., Scully, S.A., Bolon, B., Ring, B.D., Tarpley, J.E., DeRose, M., Simonet, W.S., 1998. Fgf-10 is required for both limb and lung development and exhibits striking functional similarity to *Drosophila branchless*. Genes Dev. 12, 3156–3161.

Miyake, A., Konishi, M., Martin, F.H., Hernday, N.A., Ozaki, K., Yamamoto, S., Mikami, T., Arakawa, T., Itoh, N., 1998. Structure and expression of a novel member, FGF-16, of the fibroblast growth factor family. Biochem. Biophys. Res. Commun. 243, 148–152.

Moremen, K.W., Touster, O., 1986. Topology of mannosidase II in rat liver Golgi membranes and release of the catalytic domain by selective proteolysis. J. Biol. Chem. 261, 10945–10951.

Muller, C.W., Herrmann, B.G., 1997. Crystallographic structure of the T domain–DNA complex of the Brachyury transcription factor. Nature 389, 884–888.

Nasevicius, A., Ekker, S.C., 2000. Effective targeted gene 'knockdown' in zebrafish. Nat. Genet. 26, 216–220.

Ng, J.K., Kawakami, Y., Buscher, D., Raya, A., Itoh, T., Koth, C.M., Rodriguez Esteban, C., Rodriguez-Leon, J., Garrity, D.M., Fishman, M.C., Izpisua Belmonte, J.C., 2002. The limb identity gene *Tbx5* promotes limb initiation by interacting with *Wnt2b* and *Fgf10*. Development 129, 5161–5170.

Nomura, R., Kamei, E., Hotta, Y., Konishi, M., Miyake, A., Itoh, N., 2006. *Fgf16* is essential for pectoral fin bud formation in zebrafish. Biochem. Biophys. Res. Commun. 347, 340–346.

Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N., Noji, S., 1997. The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. Development 124, 2235–2244.

Perz-Edwards, A., Hardison, N.L., Linney, E., 2001. Retinoic acid-mediated gene expression in transgenic reporter zebrafish. Dev. Biol. 229, 89–101.

Sekine, K., Ohuchi, H., Fujiwara, M., Yamasaki, M., Yoshizawa, T., Sato, T., Yagishita, N., Matsui, D., Koga, Y., Itoh, N., Kato, S., 1999. *Fgf10* is essential for limb and lung formation. Nat. Genet. 21, 138–141.

Shin, J.T., Fishman, M.C., 2002. From zebrafish to human: modular medical models. Annu. Rev. Genomics. Hum. Genet. 3, 311–340.

Takeuchi, J.K., Koshiba-Takeuchi, K., Suzuki, T., Kamimura, M., Ogura, K., Ogura, T., 2003. *Tbx5* and *Tbx4* trigger limb initiation through activation of the Wnt/Fgf signaling cascade. Development 130, 2729–2739.

Tamura, K., Yonei-Tamura, S., Izpisua Belmonte, J.C., 1999. Differential expression of *Tbx4* and *Tbx5* in zebrafish fin buds. Mech. Dev. 87, 181–184.

Westerfield, M., 2000. The Zebrafish Book. A Guide for the Laboratory Use of Zebrafish (*Danio rerio*), 4th ed. University of Oregon Press, Eugene, OR.