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ORIGINAL ARTICLE

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Zebrafish *fgfr1* is a member of the *fgf8* synexpression group and is required for *fgf8* signalling at the midbrain-hindbrain boundary

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Abstract FGFR1 is an important signalling molecule during embryogenesis and in adulthood. FGFR1 mutations in human may lead to developmental defects and pathological conditions, including cancer and Alzheimer's disease. Here, we describe cloning and expression analysis of the zebrafish fibroblast growth factor receptor 1 (*fgfr1*). Initially, *fgfr1* is expressed in the adaxial mesoderm with transcripts distinctly localised to the anterior portion of each half-somite. Hereupon, *fgfr1* is also strongly expressed in the otic vesicles, branchial arches and the brain, especially at the midbrain-hindbrain boundary (MHB). The expression patterns of *fgfr1* and *fgf8* are strikingly similar and knock-down of *fgfr1* phenocopies many aspects observed in the *fgf8* mutant *acerebellar*, suggesting that Fgf8 exerts its function mainly by binding to FgfR1.

Keywords Fibroblast growth factor receptor 1 · *Fgf8* · Midbrain-hindbrain boundary · Zebrafish · *Acerebellar*

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Introduction

Fibroblast growth factors (FGFs) comprise a large family of more than 20 proteins important for regulating cell proliferation, survival and differentiation during embryogenesis and for adult homeostasis (Szebenyi and Fallon 1999; reviewed in Ornitz and Itoh 2001). The cellular events controlled by FGFs are mediated through their interactions with fibroblast growth factor receptor proteins (FGFRs), members of the receptor tyrosine kinase superfamily (Schlessinger 2000). Five closely related *FGF receptor* genes (*FGFR1–5*) have been found in vertebrates (reviewed in Groth and Lardelli 2002). *FGFR1* plays an important role during embryonic development as well as in the adult animal. For example, *FGFR1* dysfunction has been implicated in Pfeiffer's syndrome (Passos-Bueno et al. 1999), a developmental disorder characterised by craniofacial abnormalities, and in pathological conditions including cancer and Alzheimer's disease (Takami et al. 1998; Valve et al. 2001).

The canonical view of the biological role of FGF receptors is that of cell surface receptors which, upon binding of their ligand, dimerise, leading to phosphorylation at specific cytoplasmic tyrosine residues and activation of the receptor (reviewed in Ornitz and Itoh 2001). The activated kinase phosphorylates and activates the membrane-bound FRS2 protein, allowing the recruitment of other adapter proteins to the complex and subsequent activation of the Ras/mitogen-activated protein kinase (MAPK) signalling cascade. MAPK, as target, translocates into the nucleus and phosphorylates and activates transcription factors, thereby activating transcription of target genes (Schlessinger 2000).

Fgf8 is an important signalling molecule, acting in various tissues of vertebrate embryos (Heikinheimo et al. 1994; Mahmood et al. 1995; Reifers et al. 1998). In zebrafish, *fgf8* is expressed in the neuroectoderm, more

specifically in the forebrain, the midbrain-hindbrain boundary (MHB) and rhombomere 4 (Reifers et al. 1998). Furthermore, *fgf8* expression is found in the otic vesicles, heart, limb buds, presomitic mesoderm, somites and tailbud. Functional experiments have elucidated the role of Fgf8 in anteroposterior patterning (Brand et al. 1996; Reifers et al. 1998; Picker et al. 1999; Irving and Mason 2000; Liu and Joyner 2001; Scholpp et al. 2003) as well as dorsoventral patterning (Fürthauer et al. 1997; Reifers et al. 1998). The dorsoventral aspects can be mimicked by overexpression of a constitutively activated form of zebrafish *fgfr1* (Fürthauer et al. 2001). This suggests that Fgfr1 may be a key mediator of Fgf8-dependent signalling during development.

In this study we report the isolation and characterisation of a zebrafish orthologue of human *FGFR1* (Dionne et al. 1990). Zebrafish *fgfr1* shows a strikingly similar expression pattern to *fgf8* during early development, and thus represents a novel member of the growing *fgf8* synexpression group. Morpholino-induced knockdown of *fgfr1* reveals redundant and unique functions in MHB development: target genes of Fgf8, such as *spry4*, *erm*, and *pea3* are initially affected at the MHB, and this defect persists until later stages. In addition, initiation of *pax2.1* is normal and becomes down-regulated at 24 hpf. Morphologically, lack of Fgfr1 signalling leads to a strong malformation of the MHB, whereas the tectum seems less affected. Furthermore, structures such as the otic placode are also affected. These genetical and morphological phenotypes are strikingly similar to the *fgf8* mutant *acerebellar* (Reifers et al. 1998). This suggests that Fgf8 exerts its function mainly via the activation of Fgfr1.

Materials and methods

Cloning of zebrafish *fgfr1* cDNA

A cDNA revealing a restricted pattern of embryonic gene expression similar to *fgf8* and a high degree of sequence similarity to the kinase domain of known Fgf receptor genes was isolated as part of an in situ transcript hybridisation screen (Tamme et al. 2001). This cDNA was used as a template for PCR screening of cDNA sublibraries (Lardelli 1997) made from a 9- to 16-hpf embryonic randomly primed zebrafish cDNA library in the lambda-ZAP vector (kindly provided by R. Riggleman, K. Helde and D. Grunwald) to identify positive sublibraries. Overlapping cDNA clones were isolated by plaque hybridisation from positive sublibraries using this cDNA as probe. Successive screenings using the 5′ end of isolated positive cDNA clones as probes allowed the isolation of overlapping cDNA clones covering the complete open reading frame of the Fgf receptor. Subsequently, full-length *fgfr1* transcript variants were isolated from 12-hpf cDNA by RT-PCR. Primers used for RT-PCR of full-length *fgfr1* were: forward, FGFR021F 5′-TGGAGTTCAGATGTAGAGG-3′, and reverse, FGFR022R 5′-CAGCTG TATGTGTTTCTCC-3′.

Embryos and whole-mount in situ transcript hybridisation

Zebrafish were staged according to Kimmel et al. (1995). Stages are given as equivalent to hours of development post fertilisation (hpf) at 28.5°C. The transcript expression pattern of the zebrafish *fgfr1* was analysed by whole-mount in situ hybridisation on fixed embryos at various stages of development up to 28 hpf as previously described with the modification that prehybridisation and hybridisation were performed at 70°C (Kimmel et al. 1995). A riboprobe recognising the kinase domain of *fgfr1* was synthesised by in vitro transcription from templates generated by region-specific PCR using a digoxigenin RNA labelling kit (Roche). Primers used were: forward, Kami01: 5′-CCTGCGCATATCAGGTGGC-3′; reverse, FGFR035R: 5′TAATACGACTCACTATAGGGAGGCAGCTG-TATGTGTTTCTCC-3′.

A T7 RNA polymerase promoter was included in the 5′ end of the reverse primer allowing antisense transcription using T7 RNA polymerase. Whole-mount mRNA in situ hybridisations for *spry4*, *erm*, and*pax2.1* were carried out as described by Reifers et al. (1998) and modified as described in Scholpp et al. (2003). Expression patterns have been described for *spry4* (Fürthauer et al. 2001), *erm* (Raible and Brand 2001), and *pax2.1* (Lun and Brand 1998).

Sequence and phylogenetic analysis

Human FGFR1 and zebrafish Fgfr1 protein sequences were analysed using programs available through BioManager on ANGIS (Australian National Genomic Information Service). The sequences were aligned using Clustal W (Thompson et al. 1994) and prepared for publication using Boxshade by K. Hofmann and M.D. Baron. The evolutionary relationship between metazoan *FGFR* gene sequences was analysed using Clustal W (Thompson et al. 1994) for sequence alignment and the maximum likelihood method of DNAML for generating the phylogenetic tree using the Phylip phylogeny inference package by Felsenstein (1989). The tree was prepared for publication using Phylodendron by D.G. Gilbert. The following *FGFR* gene sequences were included in the phylogenetic analysis (accession numbers are given in parentheses): human *FGFR1* (M55614), human *FGFR2* (31373), human *FGFR3* (13112046), human *FGFR4* (13112051), mouse *Fgfr1* (6753855), mouse *Fgfr2* (6753857), mouse *Fgfr3* (6679786), mouse *Fgfr4* (6679788), chick *CEK1* (M24637), chick *CEK3* (M35196), chick *CEK2* (M35195), *Xenopus FGFR1* (U2449), *Xenopus FGFR2* (64694), *Xenopus FGFR3* (2425187), *Xenopus FGFR4* (AF288453), *Pleurodeles waltl FGFR1* (64250), *P. waltl FGFR2* (396744),*P. waltl FGFR3* (414683),*P. waltl FGFR4* (64252), zebrafish *fgfr1* (this study), zebrafish *fgfr3* (AF157560), and zebrafish *fgfr4* (U23839). *Drosophila DFGFR* (285753) was chosen as the outgroup.

Mapping zebrafish *fgfr1*

Zebrafish *fgfr1* was mapped using the LN54 mouse/zebrafish radiation hybrid panel (Hukriede et al. 1999). The 5′ UTR primers used were: forward, FGFR030F 5′-CACGAGCGCAAACAAAACC-3′, and reverse, FGFR031R 5′-GCACTTTCTGAAGC AAATCC-3′. Results were analysed with RHMAPPER and placed on the zebrafish radiation hybrid map provided by ZFIN (http://zfish.uoregon.edu/ZFIN).

RT-PCR analysis

The isolation of RNA from embryos was performed using the RNeasy mini kit (Qiagen, Chatsworth, Calif.) and cDNA was generated using Omniscript Reverse Transcriptase (Qiagen) according to the manufacturer's instructions. Primers used to identify transcript variants including or missing the exons encoding the second half of the IgIII domain and the TM domain were: forward, FGFR028F 5′-CTGCCTGCAAACCGTACC-3′, and reverse, CG003R 5′-GACGGACCAACATCCCAC-3. Primers used to identify *fgfr1IIIb* variants were: forward, FGFR0025F 5′-AACTCTGGGGTCAACAGCTC-3′, and reverse, FGFR0024R 5′-

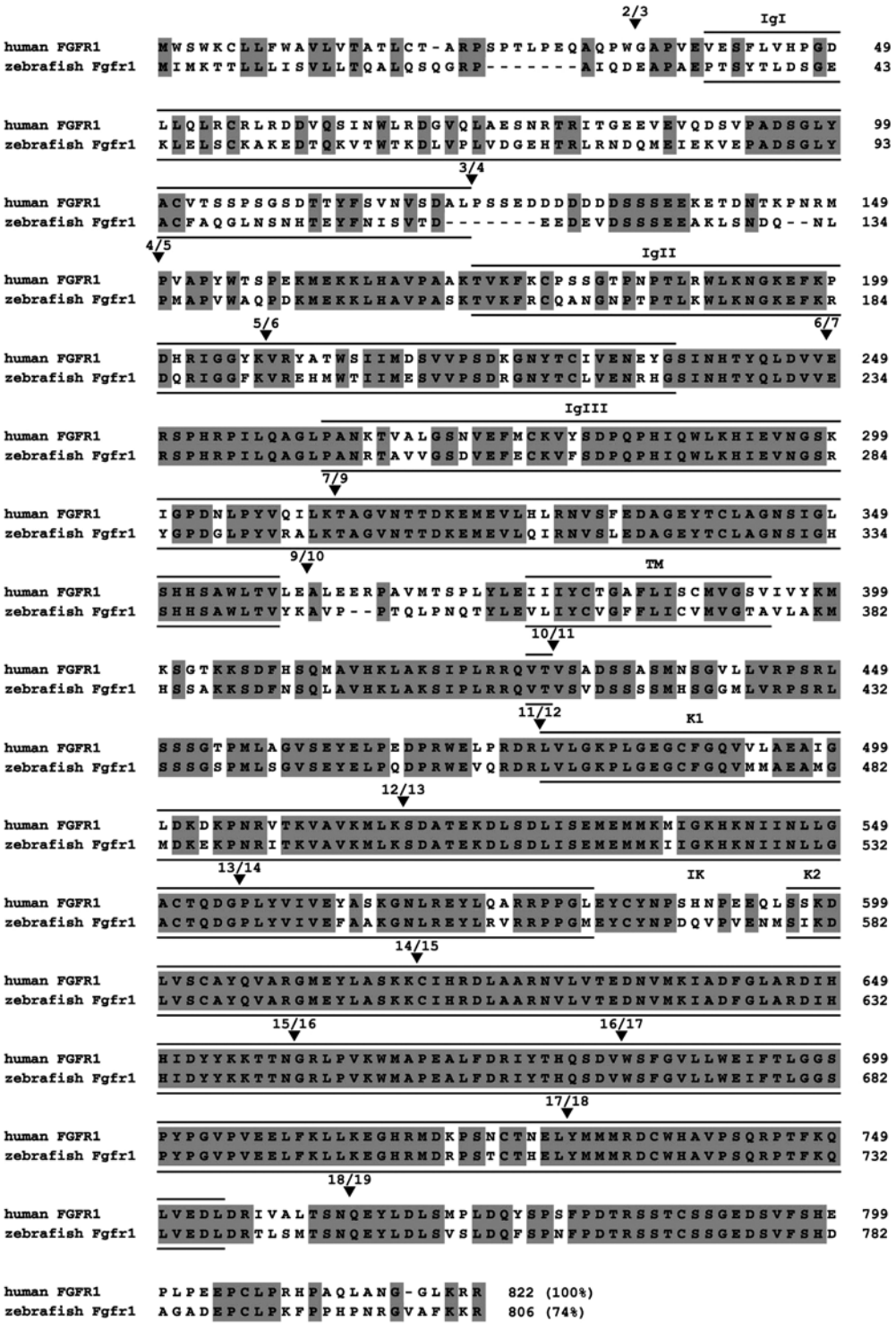

Fig. 1 Sequence alignment of human and zebrafish FGFR1 protein. Identical amino acids are *shaded*. Gaps introduced for optimal alignment are indicated by *dashes*. Structural domains identified in human FGFR1 (Johnson and Williams 1993) are marked by *lines*. *Arrowheads with numbers* indicate the exon boundaries of human FGFR1 (Cote et al. 1997; Givol and Yayon 1992; Johnson et al. 1991). *IgI–IgIII* Immunoglobulin-like domain I to III, *IK* interkinase domain, *K1* kinase domain 1, *K2* kinase domain 2, *TM* transmembrane domain

TAATACGACTCACTATAGGGAGGGTGTTTAACCACGGTGAG CC-3′. Primers used to identify *fgfr1IIIc* variants were: forward, FGFR027F 5′-ACGGCAGGCGTCAACACC-3′, and reverse, FGFR026R 5′-TAATACGACTCAC TATAGGGAGGTTATAGACAGTCAACCATGCAG-3′.

Injections

For transient knock-down of gene expression, morpholino antisense oligomers (morpholinos, MO; by GeneTools) were prepared targeting *fgfr1* as described in Scholpp et al. (2003). Morpholinos were injected into the yolk cell close to the blastomeres between the one- and eight-cell stages at a concentration of 4 ng/nl. A 4-bp mismatch Morpholino (4bpMM-MO) and a randomized mispriming Morpholino (con-MO) served as control and showed no effect on embryos injected at 15 ng/nl. Morpholino-injected

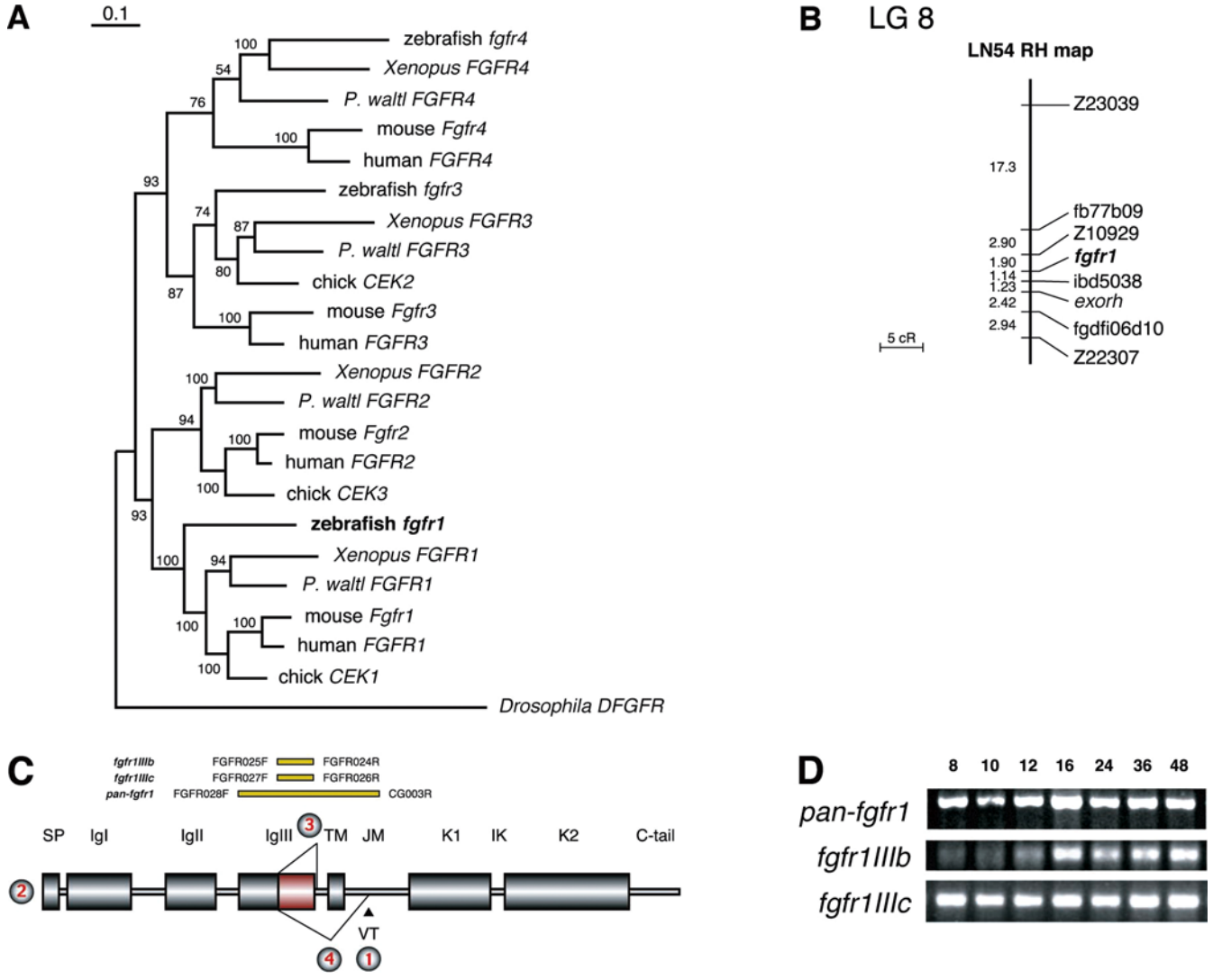

Fig. 2a–d Phylogeny, mapping and differential transcript splicing of zebrafish *fgfr1*. **a** The evolutionary relationship of zebrafish *fgfr1* to other members of the *FGFR* multigene family was established by comparison to selected metazoan *FGFR* gene sequences and the construction of a phylogenetic tree. *Drosophila melanogaster DFGFR* was chosen as the outgroup. Shown bootstrap values are based on 100 replications. **b** The map position of zebrafish *fgfr1* was assigned to LG8 using the LN54 radiation hybrid panel (Hukriede et al. 1999). **c**, **d** Alternative splicing of zebrafish *fgfr1*. **c** Schematic drawing showing the general structure of Fgfr1 and some of the predicted Fgfr1 isoforms (*1–4*). Both isoforms 1 and 2 represent Fgfr1IIIc isoforms, while isoforms 3 and 4 are lacking the C-terminal half of IgIII (*red box*). Isoform 4 lacks that the TM domain. *Yellow bars* indicate regions amplified by RT-PCR. **d** Semi-quantitative RT-PCR analysis of the temporal expression of *fgfr1* transcript variants from 8 to 48 hpf (indicated by *numbers*). *Top panel* The majority of transcripts encode putative isoforms that include the C-terminal half of IgIII; *middle panelfgfr1IIIb* transcript variants; *bottom panelfgfr1IIIc* transcript variants

embryos (so called morphants) were fixed at given stages prior to in situ hybridisation.

Sequences:

– *fgfr1* MO: 5′-gCA gCA gCg Tgg TCT TCA TTA TCA T-3′
– 4bpMM-MO: 5′-gCA g**g**A gCg **A**gg TCT **A**CA TT**T** TCA T-3′

Mismatch base pairs are marked in bold.

– con-MO: 5′-CCT CTT ACC TCA gTT ACA ATT TAT A-3′

For the rescue experiment, mRNA of the complete ORF for *fgfr1* was amplified and subcloned into the vector pCS2+ (Rupp et al. 1994) and the SP6 message machine kit (Ambion, Austin, Tex.) was used for transcription. The amount of injected mRNA was estimated from the concentration and volume of a sphere of mRNA injected into oil at the same pressure settings. mRNA was dissolved in 0.25 M KCl with 0.2% phenol red and back-loaded into borosilicate capillaries prepared on a Sutter puller. mRNA was injected into the cytoplasm of one- to two-cell-stage embryos. Typically, 125 pg *fgfr1* mRNA was injected. The embryos were fixed at appropriate stages prior to in situ hybridisation.

Results

Isolation of zebrafish *fgfr1*

Zebrafish *fgfr1* cDNA was first isolated from an in situ hybridisation screen to identify developmental control genes (Tamme et al. 2001). An approximately 800-bp cDNA showed expression similar to *fgf8* during zebrafish

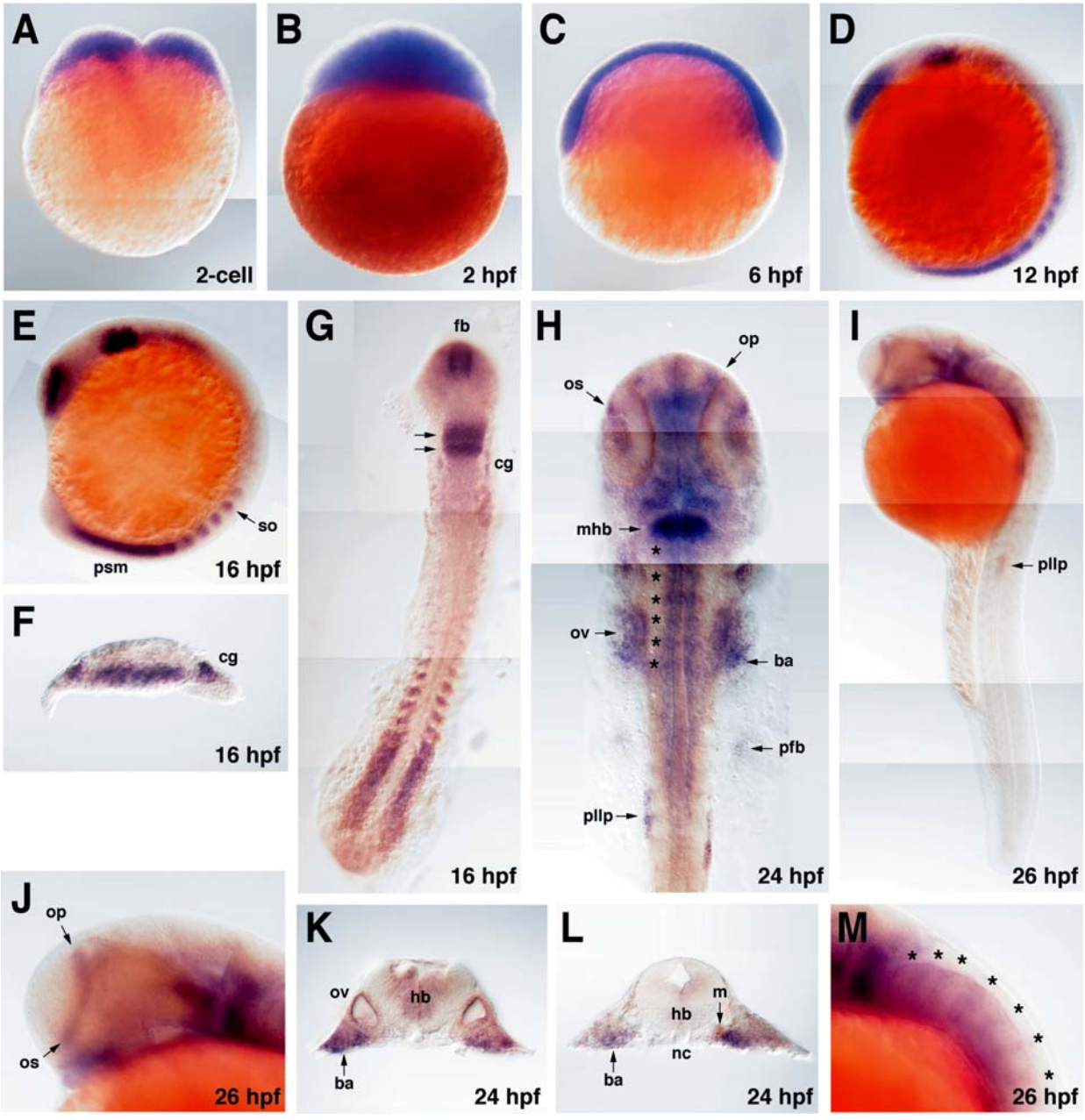

Fig. 3a–m The spatial expression of zebrafish *fgfr1* during embryogenesis. **a–c** Lateral view of embryos at 2-cell stage (**a**), 512-cell stage (**b**), 6 hpf (**c**) and 12 hpf (**d**). **e–g** Different views of the same 15-hpf embryo: **e** lateral view; **f** transverse section through hindbrain region (note that *fgfr1* is predominantly expressed in ventral mesoderm and cranial ganglia); **g** dorsal view; two stripes of *fgfr1* expression are observed in the midbrain-hindbrain region (indicated by *arrowheads*). **h**, **k**, **l** Different views of the same 24-hpf embryo: **h** dorsal view showing strong *fgfr1* expression at the MHB and segmental expression in rhombomeres (*asterisks*); **k** transversed section at the level of the otic vesicles; **l** cross-section through the post-otic region. **I**, **j**, **m** Lateral views of the same 28-hpf embryo: **I***fgfr1* staining in the migrating primordium of the posterior lateral line; **j** magnification of the head region in **I**; **m** magnification of the hindbrain region in **I** showing weak expression of *fgfr1* in a segmental pattern in rhombomeres (*asterisks*). *cg* Cranial ganglion, *ba* branchial arches, *fb* forebrain, *hb* hindbrain, *mhb* midbrain-hindbrain boundary, *m* melanocyte, *nc* neural crest, *op* olfactory placode, *os* optic stalk, *ov* otic vesicle, *pfb* pectoral fin bud, *pllp* posterior lateral line primordium, *psm* presomitic mesoderm, *so* somite. *Scale bars* indicate 100 μm, except **f**, 50 μm

embryogenesis and its amino acid sequence was highly related to that of known Fgfr kinase domains (Johnson and Williams 1993), suggesting that it encodes a region of a putative Fgf receptor. The ORF of this *fgfr* gene was assembled from the sequences of overlapping cDNA clones isolated from screening of zebrafish sublibraries (Lardelli 1997; Fig. 1).

A phylogenetic tree of vertebrate *FGFR* gene sequences shows that the zebrafish *fgfr* cDNA forms a group with vertebrate *FGFR1* genes, and thus represents a zebrafish *fgfr1* gene (Fig. 2a). Like other FGFR1 proteins the amino

acid sequence of zebrafish Fgfr1 has been highly conserved during evolution, with 81% similarity and 74% identity to human FGFR1 (Fig. 1). Alternative splicing of vertebrate *FGFR1* is extensive with approximately 20 transcript variants described so far (Groth and Lardelli 2002). Using RT-PCR with primers specific for the 5′ and 3′ UTRs of the assembled sequence, we isolated cDNAs representing complete zebrafish *fgfr1* ORFs derived from four transcript variants. The longest ORF (transcript variant 1) is 2,418 bp in length encoding a putative 806-amino-acid (type I integral) protein (Figs. 1, 2). The ectodomain of the predicted protein is 359 aa and includes three immunoglobulin-like domains (IgI–IgIII). The endodomain is 426 aa long and includes a tyrosine kinase domain split by a short interkinase region, a characteristic feature of Fgf receptor proteins (Johnson and Williams 1993). The kinase domain is preceded by the juxtamembrane domain, a region important for Fgfr-mediated signal transduction (Gillespie et al. 1995; Paterno et al. 2000). A 6-nucleotide insert encoding the dipeptide VT is found in the juxtamembrane domain of the longest ORF, but is absent in all other isolated cDNAs (Figs. 1, 2). Only the VT+ isoform has been found to interact with the FRS2 adaptor protein subsequently leading to activation of the Ras/MAPK signalling pathway (Burgar et al. 2002). In *Xenopus*, the relative levels of VT+ and VT- FGFR1 isoforms have been found to regulate mesoderm induction (Paterno et al. 2000). Transcript variant 2 is identical to transcript variant 1 except for the absence of the VT dipeptide (Fig. 2c). Alternative splicing of two exons that encode the C-terminal half of IgIII results in different isoforms (FGFR1IIIb and FGFR1IIIc) with different binding affinities for Fgfs, which has been widely reported in vertebrates (Johnson and Williams 1993). Zebrafish transcript variants 1 and 2 both encode Fgfr1IIIc isoforms. Transcript variant 3 is missing any of the alternatively spliced exons encoding the C-terminal half of IgIII while transcript variant 4 lacks the exon encoding the TM domain as well as any of the exons encoding the C-terminal half of IgIII (Fig. 2c). The sequences of these *fgfr1* transcript variants can be found in GenBank with the accession numbers AY197497–AY197500. We also isolated a partial cDNA representing a fifth transcript variant encoding a putative Fgfr1IIIb isoform. The cDNA sequence of this transcript variant has been deposited in GenBank with the accession number AY197501.

Mapping of *fgfr1*

Zebrafish *fgfr1* was mapped using the LN54 radiation hybrid panel (Hukriede et al. 1999) to linkage group 8 at 59.97 cR from the marker Z1052 with a LOD of 11.0 (Fig. 2b; the second best linkage score was a LOD of 5.7 to a marker in linkage group 12).

Developmental expression of *fgfr1* transcript

The expression pattern of *fgfr1* during zebrafish development was analysed by whole-mount in situ hybridisation (Fig. 3). The riboprobe used is specific for the kinase domain and, consequently, the shown patterns represent the sum of all transcript variants that include this region. Maternally derived *fgfr1* mRNA is found in cleavage stage blastomeres (Fig. 3a, b). Strong ubiquitous expression is present during gastrulation (Fig. 3c). During the segmentation stage high-level expression of *fgfr1* is found in the forebrain, in the region of the MHB, presomitic mesoderm and somites (Fig. 3d–g). Weak expression is also present at this stage in ventral mesoderm (Fig. 3f, g). At 12 hpf, expression in the MHB region is present as one broad stripe (Fig. 3d), which by 15 hpf is clearly split into two separate domains (Fig. 3e). Expression is also found in the cranial sensory ganglia at 15 hpf (Fig. 3f, g). During late segmentation and early pharyngeal stages strong expression is found in domains of the telencephalon and ventral diencephalon including the olfactory placode and optic stalk (Fig. 3h–j). In the midbrain, the tegmentum shows a high level of *fgfr1* transcription. Expression in the MHB region is especially strong. In the hindbrain *fgfr1* transcripts are found in the otic vesicles and branchial arches (Fig. 3h–m). A weaker but distinct metameric *fgfr1* pattern is present in the hindbrain (Fig. 3i, m). Expression is also present in the pectoral fin buds and the migrating primordia of the posterior lateral line (Fig. 3h, i). In order to determine the temporal developmental expression pattern of some of the isolated transcript variants we performed variant-specific RT-PCR on cDNA isolated from different developmental stages ranging from 8 to 48 hpf (Fig. 2). We have shown that the predominant transcript species contains the C-terminal half of IgIII, i.e. *fgfr1IIIb* or *fgfr1IIIc* variants. *fgfr1IIc* was expressed during all stages examined whereas *fgfr1IIIb* expression became detectable from 12 hpf onwards (Fig. 2d).

Functional knock-down of Fgfr1 copies many aspects of the acerebellar phenotype

In zebrafish embryos, Fgf8 is expressed at the MHB and is required for its formation as shown by the phenotype of *acerebellar* (*ace*) mutations in which this gene is defective (Brand et al. 1996; Reifers et al. 1998). *fgfr1* is the only known Fgf receptor gene expressed at high levels in the MHB. If Fgfr1 is required for transmission of Fgf signals patterning the MHB, then loss of Fgfr1 function would result in loss of MHB-derived structures. To observe the gene regulatory and morphological consequences of loss of Fgfr1 function, we generated "*fgfr1* morphants" by reducing translation of *fgfr1* mRNA using antisense morpholino oligonucleotides (Nasevicius and Ekker 2000). The genetic and morphological consequences of loss of Fgfr1 activity are shown in Figs. 4 and 5.

To examine whether Fgfr1 is the primary transducer of Fgf signals in the MHB, we examined the expression of

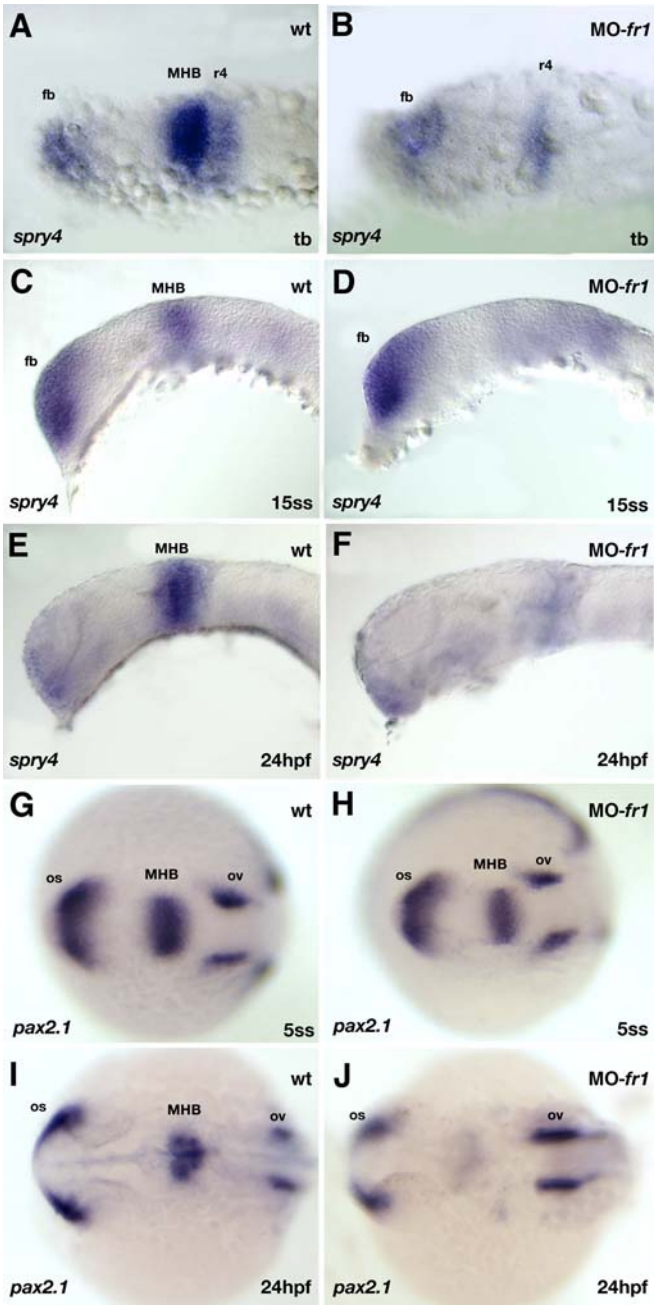

Fig. 4a–h Knock-down of Fgfr1. Analysis of *spry4* expression (**a**–**f**) and *pax2.1* expression (**g**–**j**) at the indicated stages. **a***spry4* is expressed in forebrain, MHB, and rhombomere 4 at tailbud stage. Expression domains in forebrain and MHB persist until the 15 somite stage (ss) and 26 hpf (**c**, **e**). At 15 ss a novel expression domain at the otic placode is detectable (**c**). In *fgfr1* morphant embryos, the expression domain at the MHB is downregulated (**b**) and is hardly detectable at older stages (**d**, **f**), whereas the expression in forebrain and otic vesicles is not affected. Expression of *pax2.1* is not affected at the 5 s stage (**g**, **h**); *pax2.1* is strongly down-regulated at the MHB at 24 hpf. The expression in the otic placode, as well as in the otic stalk, looks similar (**I**, **j**). *fb* Forebrain, *MHB* midbrain-hindbrain boundary, *os* otic stalk, *ov* otic vesicle, *r4* rhombomere, *tel* telencephalon

genes that are known to modulate, and are modulated by, Fgf signalling. *Sprouty4* (*spry4*) has been shown to be regulated by Fgf8 and Fgf3 (Fürthauer et al. 2001). *spry4* interacts with signals from Fgfr1 and can inhibit the activity of Fgf8 and Fgf3. Reduction of Fgfr1 expression in *fgfr1* morphants resulted in down-regulation of *spry4* transcription at the MHB at tailbud stage (10 hpf, *n* =39/67; Fig. 4a, b). Interestingly, the other expression domains of *spry4* in the forebrain and in rhombomere 4 were only weakly affected, suggesting that other Fgf receptors are contributing to the maintenance of *spry4* expression in these regions or that another Fgf, presumably Fgf3, has the ability to exert its function via a different Fgf receptor. At 16.5 hpf (15 somite stage; 15 ss) and 26 hpf, the *spry4* expression domain at the presumptive MHB is strongly affected in *fgfr1* morphants (Fig. 4c–f; *n* =43/78). To examine development of the MHB territory in *fgfr1* morphant embryos further, we analysed the expression of the Fgf8 independent marker *pax2.1* (Reifers et al. 1998). At 11.6 hpf (5 ss), expression of *pax2.1* is not affected in the region of the optic stalk, the MHB territory and the otic vesicle in *fgfr1* morphant embryos (Fig. 4g, h). At 24 hpf, in the maintenance phase of the MHB, *pax2.1* expression is down-regulated at or even absent from the MHB (Fig. 4I, j; *n* =34/61).

Expression of the ETS-domain transcription factor gene *erm* is tightly controlled by Fgf signalling and rapidly responds to changes in levels of Fgf8 and Fgf3 (Raible and Brand 2001; Roehl and Nüsslein-Volhard2001). From the time of its onset in the MHB, the expression of *erm* in *fgfr1* morphants is affected in a manner similar to that of *spry4* (data not shown). At mid-somitogenesis (5 ss), *erm* expression at the MHB is absent and the hindbrain expression domain is slightly down-regulated (Fig. 5a–b′; *n* =41/63). At 16.5 hpf (15 ss), *erm* expression is still absent at the MHB (Fig. 5e–f′), while *erm* is expressed at reduced levels in the neural crest and the otic vesicles. The absence of *erm* expression from the MHB is similar to that seen in the Fgf8 mutant *acerebellar* (Fig. 5b, c, f, g). *erm* expression is affected in a similar manner in placodal structures of the otic vesicles and neural crest of both the *fgfr1* morphant and *ace* embryos (Fig. 5b′, c′, f′, g′). A very similar observation was made with another down-stream factor of Fgf8, *pea3* (data not shown).

The dramatic effect of loss of Fgfr1 activity on *erm* expression allowed us to test the specificity of the *fgfr1* MO. We synthesised *fgfr1* mRNA lacking complementarity to the *fgfr1* MO and co-injected this with the lineage tracer rhodamine dextran (MW 2,000,000) into one blastomere of a two-cell stage embryo. At the four-cell stage, we subsequently injected the *fgfr1* MO. The morpholino oligonucleotide is able to spread to all cells of the embryo through cytoplasmic channels that exist in early cleavage stage embryos (Kimmel et al. 1995). However, the larger mRNA and dye molecules remain confined to one side of the embryo derived from the corresponding blastomere. At the 5 and 15 somite stages we observed unilateral loss of *erm* transcript on one side of embryos and rescue of this phenotype on the *fgfr1* mRNA-injected, rhodamine-dextran-labelled side, visible by expression of *erm* at the MHB or the size of the otic placode (Fig. 5d, h, marked by asterisk; *n* =17/43).

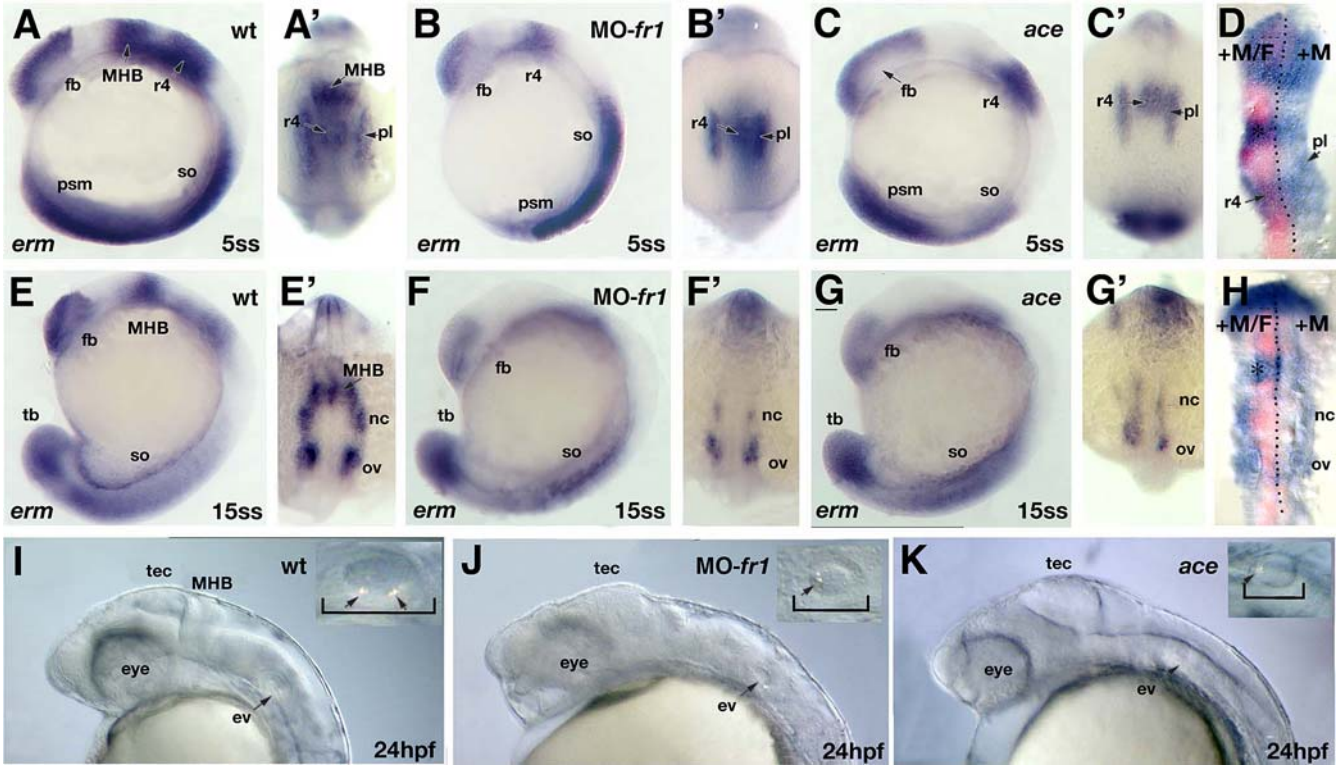

Fig. 5a–h *fgfr1* morphant embryos phenocopy the acerebellar mutant phenotype. At the 5 somite stage (ss), expression of *erm* is seen in the neuroectoderm: in forebrain, MHB, rhombomere 4, somites, presomitic mesoderm (**a**) and in the otic placode (**a′**). The expression domains are still detectable at the 15 ss (**e**, **e′**). In *fgfr1* morphant embryos, the expression domain at the MHB is not detectable (**b**, **b′**, **f**, **f′**). Forebrain expression is down-regulated, whereas expression in the tailbud and the somites is not altered (**f**). In addition, the otic placodal expression as well as expression in the neural crest is reduced from 5 ss onwards (**b′**, **f′**). Furthermore, the body axis is shortened in size, suggesting a cell migration defect (**b**), which recovers at the 15 ss (**f**). The changes in marker gene expression in *fgfr1* morphants are similar to the phenotype observed in the *ace* mutant (**c**, **c′**, **g**, **g′**). To rescue the morphant phenotype, non-inhibitable *fgfr1* mRNA was co-injected with rhodamine-dextran at the two cell stage into one blastomere. An overlay picture of the rescued phenotype is shown in **d** and **h**. + *M* marks the morphant side, whereas the + *M/F* marks the rescued side, co-injected with *fgfr1* mRNA and lineage tracer. *Asterisks* mark the position of MHB expression, the *dotted line* marks the midline. In the + M/F side the expression domain at the MHB is reduced (**d**, **h**) and the size of the otic placode is similar to wt siblings (**h**). At 26 hpf, in *fgfr1* morphants the structure of the MHB is severely affected. Similar to the ace phenotype, the size of tectum increases. In addition, the otic placode shrinks and only one otolith (marked by *arrows*) is detectable, comparable to *acerebellar* mutants (shown in *insets*). *ev* Ear vesicle, *eye* eye vesicle, *fb* forebrain, *MHB* midbrain-hindbrain boundary, *nc* neural crest, *ov* otic vesicle, *pl* placode, *psm* presomitic mesoderm, *r4* rhombomere 4, *tec* tectum

We also compared wt siblings, *fgfr1* morphants and *acerebellar* mutants anatomically. In *fgfr1* morphants, the body axis is shortened during mid-somitogenesis stages but recovers to its normal length at the 15 ss (Fig. 5b, f). At 28 hpf, MHB-derived structures such as the cerebellar primordium are largely absent and the tectum appears to be enlarged, consistent with the *ace* phenotype (Fig. 5i–k; Reifers et al. 1998; Jaszai et al. 2003). The tectum is present while the otic vesicle is decreased (compare Fig. 5i, j). Within the otic vesicles, loss of one otolith is frequently observed in the *fgfr1* morphants (n =31/74; Fig.5i, j, insets) and in *ace* embryos (Fig. 5k; Leger and Brand 2002).

The exclusive expression of Fgfr1 at the MHB, together with similar morphological changes and effects on marker gene expression observed upon loss of Fgf8 and Fgfr1 activity, strongly support the idea that Fgfr1 is the main receptor for Fgf8 signalling in the MHB of zebrafish embryos.

Discussion

Evolution of *fgfr1*

We have isolated a zebrafish fibroblast growth factor receptor with high similarity to other vertebrate FGFR proteins. Phylogenetic analysis (Fig. 1) has suggested that this zebrafish *fgfr* gene is an orthologue of human *FGFR1*. Consequently, we named this zebrafish fibroblast growth factor receptor *fgfr1*. Human FGFR1 shows 74% amino acid identity and 81% similarity to zebrafish Fgfr1 and the kinase domain is 94% identical between these two distantly related species (Fig. 2), suggesting that FGFR1 controls important functions conserved during the evolution of the divergent lineages leading to extant fish and mammals. This idea is also supported by our results, which demonstrate significant similarity of embryonic *fgfr1* expression patterns in zebrafish and other vertebrates. For example, elevated *fgfr1* expression at the MHB is found in zebrafish and *Xenopus* (Golub et al. 2000). In

addition, segmented expression of *FGFR1* in presomitic mesoderm and somites has also been reported in mouse and chick (Yamaguchi et al. 1992; Walshe and Mason2000). We have also found that *fgfr1* was expressed in the pectoral fin buds (Fig. 4), indicating that zebrafish *fgfr1* may play a role in limb bud development similar to that shown in mouse (Partanen et al. 1998; Peters et al. 1992). A prominent feature of FGFR1 biology is the extensive alternative splicing, generating proteins with potentially different cellular functions (Groth and Lardelli 2002). In this study, we have identified five different zebrafish *fgfr1* transcript variants (Fig. 2), showing that, like in mammals, alternative splicing generates multiple isoforms to control various aspects of Fgfr1 function.

fgfr1 is the only fgfr strongly expressed at the MHB

The expression patterns of *fgf8* and *fgfr1* are strikingly similar (Fig. 4; Reifers et al. 1998) which suggest that Fgfr1 is a receptor for Fgf8-mediated signalling in zebrafish. Several genes of the *fgf8* synexpression group have been demonstrated to function in the FGF signalling pathway and are often found to be co-regulated. For example, expression of the FGF signalling antagonists*sef* and *sprouty4* are positively regulated by *fgf8* (Fürthauer et al. 2001, 2002; Tsang et al. 2002). We have shown that *fgfr1* is abundantly expressed at the MHB (Fig. 4), while none of the three other zebrafish *fgfr* genes shows expression at the MHB (Thisse et al. 1995; Slepsova-Friedrich et al. 2001; Tsang et al. 2002). This suggests that Fgfr1 is the receptor that is responsible for the transduction of Fgf8 signals at the MHB. The details of this interaction remain to be investigated in the future.

Knock-down of *fgfr1*reveals high similarity with the *fgf8* mutant *acerebellar*

In a functional analysis of Fgfr1, we observe that embryos in which translation of Fgfr1 is blocked display a high similarity to *ace* mutant embryos. First, Fgf-dependent target genes like *spry4*, *erm* and *pea3* are reduced in their expression levels. Second, the early expression of Fgf-independent marker genes like *pax2.1* is not affected in the MHB territory. This suggests that MHB fate has not been changed globally at early somitogenesis stages. In the maintenance phase of the MHB, however, a loss of *pax2.1* expression is observed, arguing that the MHB area has adopted midbrain fate at 24 hpf. Also similar to *ace*, the structure of the MHB is missing and the otic vesicles are reduced (Reifers et al. 1998; Leger and Brand 2002). Interestingly, the tectum seems to be enlarged posteriorly, a phenotype recently described for *ace* (Jaszai et al. 2003).

In addition to the *ace*-like characteristics, we find a shortening of the body axes in *fgfr1* morphants. This axial shortening recovers at late somitogenesis stages, suggesting an early function of Fgfr1 in mesoderm specification and/or cell migration. Interestingly, similar phenotypes were observed in *Xenopus* injected with a dominant negative Fgf receptor construct, in which mesoderm induction is altered (Amaya et al. 1993) and in *Fgfr1*$^{-/-}$ mutant mice in which mesodermal cells fail to migrate from the streak region (Deng et al. 1995; Yamaguchi et al. 1995; Ciruna and Rossant 2001). One explanation for the difference to the *ace* mutant could be that another early Fgf transduces its signal via Fgfr1. One potential candidate could be Fgf4, because in *Xenopus* global Fgf signalling is affected in the injection assay and more specifically Fgfr1$^{-/-}$ mutant mice lack Fgf4 expression in the primitive streak (Sun et al. 1999). Additional expression and genetic analysis will need to be carried out to test if Fgf4 expression is altered in zebrafish *fgfr1* morphant embryos.

Recently, an MHB-specific conditional knock-out of the FGFR1 gene was described in mice (Trokovic et al. 2003). As in zebrafish, expression of FGF-dependent markers, e.g. *Sprouty1*, are reduced in these mice. Interestingly, the onset of *Spry1* expression was not altered. In addition, the medial part of the cerebellum (vermis) is lost. The lateral parts (cerebellar hemispheres), however, are still present, but malformed. These observations do not reflect the situation observed in the *fgfr1* morphants or *ace* mutant embryos. This slight difference could be explained by expression of a different FGF receptor, which is able to functionally compensate for Fgfr1. Furthermore, residual FGFR1 activity may persist in these embryos due to incomplete gene knock-out by the used “cre-flox” system, leading to a normal onset of Fgf-dependent target gene expression and the resulting “pre-specification” of the cerebellar anlage as reflected by the presence of cerebellar hemispheres.

Remarkably, like the *fgfr1* morphants, midbrain and MHB phenotype of FGFR1 knock-out mice display a higher similarity to Fgf8 mutant fish than FGF8 mutant mice. A midbrain-specific conditional FGF8 knock-out in mice shows a strong increase in cell death and eventual elimination of the midbrain (Chi et al. 2003). Because midbrain development is likely to be initiated in these mice, pre-specified midbrain cells may undergo cell death. Alternatively, FGFR1 may not be the only receptor for FGF8 acting in midbrain development in mice.

Our analysis provides evidence that in zebrafish, Fgf8 exerts its function mainly via Fgfr1.

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