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Xenopus laevis FGF receptor substrate 3 (*XFrs3*) is important for eye development and mediates *Pax6* expression in lens placode through its Shp2-binding sites

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ABSTRACT

Members of the fibroblast growth factor (FGF) family play important roles during various developmental processes including eye development. FRS (FGF receptor substrate) proteins bind to FGFR and serve as adapters for coordinated assembly of multi-protein complexes involved in Ras/MAPK and PI3 kinase/Akt pathways. Here, we identified *Xenopus laevis Frs3* (*XFrs3*), a homolog of vertebrate *Frs3*, and investigated its roles during embryogenesis. *XFrs3* is expressed maternally and zygotically with specific expression patterns throughout the early development. Knockdown of *XFrs3* using a specific antisense morpholino oligonucleotide (MO) caused reduction of *Pax6* expression in the lens placode, and defects in the eye ranging from microphthalmia to anophthalmia. *XFrs3* MO-induced defects were alleviated by wild type XFrs3 or a mutant XFrs3 (XFrs3-4YF), in which the putative tyrosine phosphorylation sites served as Grb2-binding sites are mutated. However, another XFrs3 mutant (XFrs3-2YF), in which the putative Shp2-binding sites are mutated, could not rescue the defects of *XFrs3* morphants. In addition, we found that *XFrs3* is important for FGF or IGF-induced ERK activation in ectodermal tissue. Taken together, our results suggest that signaling through Shp2-binding sites of XFrs3 is necessary for the eye development in *Xenopus laevis*.

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Introduction

Fibroblast growth factors (FGFs) constitute a large family of polypeptide growth factors and they have been implicated in diverse cellular processes (Dailey et al., 2005). During embryonic development, FGF signaling plays important roles in the induction and maintenance of mesoderm and neural ectoderm, the control of morphogenetic movements, anterior-posterior patterning, somitogenesis, and the development of various organs (Böttcher and Niehrs, 2005; Dorey and Amaya, 2010). In mouse, *Xenopus laevis* and zebrafish, disruption of FGF signaling by *FGF receptor* (*Fgfr*) knockout or by overexpression of a dominant negative FGFR1 mutant (XFD) severely disrupts body axis formation and causes defects in trunk and tail structures (Amaya et al., 1991; Deng et al., 1994; Yamaguchi et al., 1994; Griffin et al., 1995). In addition, activin-mediated mesoderm induction in *Xenopus* animal cap cells is blocked by XFD, indicating that FGF signaling is required for cells to respond to TGFβ-like mesoderm inducing signals (LaBonne and Whitman, 1994; Cornell et al., 1995). In zebrafish embryos, activation of FGF8/FGFR1/Ras signaling pathway leads to the expansion of dorsolateral derivatives at the expense of ventral and posterior domains by an early inhibition of the ventral expression of bone morphogenetic proteins (BMPs) (Fürthauer et al., 2001, 2002). FGFs are also sufficient to induce the expression of pre-neural markers, and inhibition of FGF signaling blocks neural induction in chick embryo (Storey et al., 1998; Streit et al., 2000).

FGF ligands induce biological responses by binding to and activating FGFRs. Formation of FGF-heparin(HSPG)–FGFR complex leads to the receptor auto-phosphorylation, and the activation of intracellular signaling cascades including the Ras/MAPK pathway, the phosphatidylinositol 3 (PI3) kinase/Akt pathway and the phospholipase Cγ (PLCγ)/Ca^{2+} pathway (Böttcher and Niehrs, 2005; Dorey and Amaya, 2010). Tyrosine auto-phosphorylation

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of FGFRs controls the assembly of signaling complexes (Schlessinger, 2000). Phosphorylated tyrosine residues in FGFRs function as binding sites for Src homology 2 (SH2) and phosphotyrosine binding (PTB) domains of signaling proteins, resulting in their phosphorylation and activation (Pawson et al., 1993; Forman-Kay and Pawson, 1999). Among FGFR binding proteins, some of them such as Src, Crk and PLCγ, possess intrinsic catalytic activities whereas others, for instance FRS proteins, simply serve as the docking sites for downstream effectors (Mohammadi et al., 1991; Zhan et al., 1994; Larsson et al., 1999; Ong et al., 2000).

FRS2 (FGF receptor substrate 2), also known as FRS2α or SNT1 (suc1-associated neurotrophic factor target 1), is a myristoylated docking protein (Kouhara et al., 1997). FRS2 contains an N-terminal PTB domain and multiple FGF-induced tyrosine phosphorylation sites, which serve as the binding sites for SH2 domains of Grb2 and Shp2 (Wang et al., 1996; Hadari et al., 1998). FRS2 constitutively binds to FGFR1 and, after the activation of FGFR, tyrosine-phosphorylated FRS2 functions as a site for coordinated assembly of multi-protein complexes activating and controlling Ras/MAPK and PI3 kinase/Akt pathways (Wang et al., 1996; Kouhara et al., 1997; Xu et al., 1998; Ong et al., 2000; Hadari et al., 2001). FRS2 plays important roles during development. *Frs2*$^{-/-}$ mouse embryos die at E7.0 - 7.5 with defects in extra-embryonic development (Hadari et al., 2001; Gotoh et al., 2005). Mouse chimera and *Xenopus* knockdown studies have implicated *FRS2* in convergent extension and mesoderm development, respectively (Akagi et al., 2002; Gotoh et al., 2005). In *Xenopus* embryos, overexpression of an XFrs2 mutant, in which the FGF-induced tyrosine phosphorylation sites are mutated, interferes with mesoderm patterning while ectopic expression of XFrs2 induces mesoderm in animal cap explants (Hama et al., 2001; Kusakabe et al., 2001). Gotoh et al. (2004a) have shown that *Frs2α* mutant (*Frs2α*$^{2F/2F}$) mice, in which the two tyrosine residues serving as Shp2-binding sites are mutated, exhibit variably penetrant defects in eye development.

FRS3 (also known as FRS2β or SNT2) is the second member of the FRS family (Xu et al., 1998; McDougall et al., 2001; Zhou et al., 2003). FRS2 and FRS3 share a similar domain composition: an N-terminal myristoylation sequence (MGXXXS/T), a PTB domain and multiple tyrosine phosphorylation sites. Analyses of the expression patterns of *Frs2* and *Frs3* in mouse embryos reveal that *Frs3* is expressed predominantly in developing neural epithelium while *Frs2* is expressed in multiple tissues (McDougall et al., 2001; Gotoh et al., 2004b). However, the similarity of amino acid sequences and structural domains suggest overlapping functions of FRS2 and FRS3 in mediating cellular responses. Like FRS2, FRS3 interacts with both FGF and Trk receptors, and overexpression of FRS3 in *Frs2* null fibroblasts reconstitutes MAPK activation by FGF (Xu et al., 1998; Ong et al., 2000; Gotoh et al., 2004b). Nevertheless, the *in vivo* functions of *FRS3* during development are still largely unknown.

Here, we identified *Xenopus laevis Frs3* (*XFrs3*) and examined its roles during development. *XFrs3* is expressed ubiquitously before gastrulation and, after the onset of neurulation, its expression becomes restricted to somites and anterior structures of neuroectodermal origin (brain, eyes, branchial arches and otic vesicles). Antisense morpholino-mediated knockdown of *XFrs3* caused defects in eye, and analyses of marker gene expression indicate that *XFrs3* is involved in lens placode formation. In addition, we found that an XFrs3 mutant (XFrs3-2YF), in which the two putative Shp2-binding tyrosine residues are mutated, could not alleviate the eye defects caused by *XFrs3* knockdown, while wild-type XFrs3 and another XFrs3 mutant (XFrs3-4YF), in which the four putative Grb2-binding sites are mutated, could do so. Finally, we present evidences that *XFrs3* is important for the activation of ERK by FGF or IGF in ectodermal tissue. These results suggest that intracellular signaling through the Shp2-binding sites in XFrs3 is essential for eye formation in *Xenopus laevis*.

Materials and methods

Identification and subcloning of XFrs3

To identify potential members of *Frs* in *Xenopus laevis*, cDNA was PCR amplified with following degenerate primers: forward 5′- (A/T)AN TT(T/C) TGG AG(T/C) CTN GAN (T/C)T -3′ and reverse 5′- A(T/A)N GGN CG(G/A) AA(G/A) AAN A(A/G)N TC -3′. cDNA was synthesized with total RNA from *Xenopus laevis* stage 15 embryos, adult liver and spleen. Resulting PCR products were cloned to pCR2.1-TOPO (Life Technologies). Database search with sequences of cloned cDNAs identified an EST clone containing full-length open reading frame (ORF) of *Xenopus laevis Frs3* (GenBank accession number BC071027). This IMAGE clone was used for subsequent analyses. Expression plasmids for C-terminal Flag-tagged XFrs3 are constructed in pCS2+ vector, and only the ORF was subcloned to avoid targeting by *XFrs3* MO (see below). Following tyrosine residues in XFrs3 were substituted to phenylalanine by PCR-based mutagenesis: XFrs3-4YF for Tyr191, Tyr305, Tyr350 and Tyr398; XFrs3-2YF for Tyr445 and Tyr480; XFrs3-6YF for Tyr191, Tyr305, Tyr350, Tyr398, Tyr445 and Tyr480.

Morpholino antisense oligonucleotides

All morpholino antisense oligonucleotides (MO) were purchased from Gene Tools, LLC. The sequence of *XFrs3* MO is 5′- TAT CAG CAC ATC AGG TGA CTT CTT G -3′, and it is complimentary to 5′ UTR of *XFrs3*. Standard MO and random MO were used as negative controls.

Manipulation and microinjection of Xenopus laevis embryos

Capped mRNA was synthesized using SP6 or T7 mMESSAGE mMACHINE Kit (Ambion). Fertilized eggs were injected with mRNA and maintained in 0.33 × MMR. For lineage tracing, mRNA for nuclear β-galactosidase was co-injected. Animal cap explants were maintained at 16 °C in 0.7 × MMR. Recombinant human FGF basic and IGFII proteins (R&D Systems) were used to treat animal cap explants. Use of *Xenopus laevis* was approved by the IACUC of Ewha Womans University.

Whole-mount in situ hybridization, lineage tracing and histological analysis

For whole-mount *in situ* hybridization and histological analysis, embryos were fixed in MEMFA (3.7% formaldehyde, 100 mM MOPS, 2 mM EGTA, 1 mM $MgSO_4$) and subjected to serial dehydration in methanol. *In situ* hybridization was performed using digoxigenin-labeled RNA probes as previously described with minor modifications (Harland, 1991). 3′ UTR of *XFrs3* is used as a template for synthesizing probes. For lineage tracing, embryos were first stained with Red-Gal (Research Organics) for β-galactosidase activity and subsequently processed for *in situ* hybridization. For cryosection of stained embryos, embryos were equilibrated in 1.6 M sucrose/PBS for 12 h at 4 °C, embedded in OCT compound (Miles), and cryosectioned at 10 μm thickness. Embryo sections were mounted on slides, dried, and then cleared for photography with HistoChoice clearing Agent (Sigma-Aldrich) after serial dehydration. For histological analysis, fixed embryos were subjected to serial dehydration and rehydration in ethanol, and several washes in xylene. Embryos were embedded in Histosec pastilles (Merck), sectioned at 7 μm thickness, and stained with hematoxylin/eosin (Merck).

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