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Molecules and Cells

Fgfbp1 Is Essential for the Cellular Survival during Zebrafish Embryogenesis

Hae-ock Lee*, Hyerim Choe, Kyungwoon Seo, Hyunsook Lee, Jinseon Lee[1], and Jhingook Kim[2]

Fibroblast growth factor binding protein 1 (FGFBP1) is expressed in various tumors and may serve as a diagnostic marker and/or a therapeutic target. Previous studies suggested FGFBP1 functions as an angiogenic switch molecule by regulating the activity of FGF2, and it was later found to associate with a broad spectrum of FGFs. To study FGFBP1, we used zebrafish, in which the function of extracellular matrix protein can be easily studied in intact tissues or organisms. When Fgfbp1 expression was knocked down, morphants manifested massive cell death and structural abnormalities. Cell death was most prominent in the brain and the neural tube, but not limited to those regions. These findings suggest that the primary function of Fgfbp1 may be to sustain cellular survival throughout embryogenesis. For comparison, the expression of *fgf2* was limited to the early stage of embryogenesis and *fgf2* morphants showed more severe phenotype, with high morbidity before reaching 14-somites. Taken together, our work reveals the physiologic function of Fgfbp1, and that its function could be exerted in a Fgf2-independent manner.

INTRODUCTION

FGFBP1 (Abuharbeid et al., 2006) is up-regulated in many types of cancer cells, including colon, breast, and squamous cancers (Czubayko et al., 1997; Kagan et al., 2003; Kurtz et al., 1997; Ray et al., 2003). The functional significance of FGFBP1 expression is well documented, as its over-expression has been shown to promote tumor cell growth and vascularization in transplanted tumors (Czubayko et al., 1994). Conversely, FGFBP1 depletion diminished tumor growth and angiogenesis (Czubayko et al., 1997). These studies suggest that FGFBP1 up-regulation during tumor progression has clinical importance and warrant comprehensive analyses of this molecule.

FGFBP1 may promote cellular growth and angiogenesis by mobilizing FGFs and enhancing their activity (Aigner et al., 2001; Tassi et al., 2001). The best studied molecule for this function is FGF2, which is secreted and bound to extracellular matrix (ECM) components. Stored FGF2 can be released to activate FGF receptors and elicit biological activities. This release may occur after digestion of ECM components through the action of heparinases and proteinases (Damon et al., 1989; Sommer and Rifkin, 1989). Alternatively, FGFBP1 may bind to FGF2 molecules and transport them to FGF receptors (Wu et al., 1991). The C-terminus of FGFBP1 was mapped as the FGF2 interacting domain, and the heparin binding domain was mapped to the middle (Wang et al., 1998; Xie et al., 2006). These regions are highly conserved among species. Besides mobilizing ECM-bound FGF2, FGFBP1 may directly affect FGFR signaling, as shown by MAPK phosphorylation in cultured cell lines (Xie et al., 2006). Therefore, FGFBP1 is an important modulator of FGF2-FGFR signaling, and considering the potential interaction of FGFBP1 with other FGF family members (Beer et al., 2005), FGFBP1 may function as a universal regulator for FGF-FGFR signaling.

As FGF family members play vital roles during development, FGFBP1 is likely to be involved in the developmental process. Here, we applied the advantages of the zebrafish system to investigate the developmental role of Fgfbp1. We found that Fgfbp1 is highly expressed in the zebrafish throughout embryogenesis. Knock-down of Fgfbp1 induced massive apoptosis in the head and trunk, and caused structural abnormalities. By comparison, its partner Fgf2 had limited expression time, and the morphants manifested prominent cell death at an early stage of embryogenesis. Altogether, our results demonstrate that Fgfbp1 is required for cellular survival throughout zebrafish embryogenesis, even in the absence of Fgf2.

MATERIALS AND METHODS

Morpholino (MO) and RNA injections

Zebrafish *fgfbp1* and *fgf2* morpholinos targeting the ATG start codon were obtained from GeneTools (USA). Splice site targeting MOs were unavailable. MO sequences are CGCATCATGGCTGCATTGGAAATAC for *fgfbp1* and GGCCATCCCTAAGTCTGTCGGTCTC for *fgf2*. As a control standard MO and 5-mismatch MO for *fgfbp1* (CGgATCATcGCTcCATTcGAAAaAC) were utilized. MO's were diluted in nuclease free water and injected into naturally spawned embryos at 1-4 cell stages. For the rescue experiments, *fgfbp1* or *gfp* RNAs were transcribed from each gene cloned in pCS2 using mMessage

Department of Biological Sciences and Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea, [1]Department of Thoracic Surgery, Samsung Medical Center, Sungkyunkwan University School of Medicine, Seoul 135-710, Korea, [2]Cancer Research Division, Center for Clinical Research, Samsung Biomedical Research Institute, Seoul 135-710, Korea
*Correspondence: haeockl@snu.ac.kr

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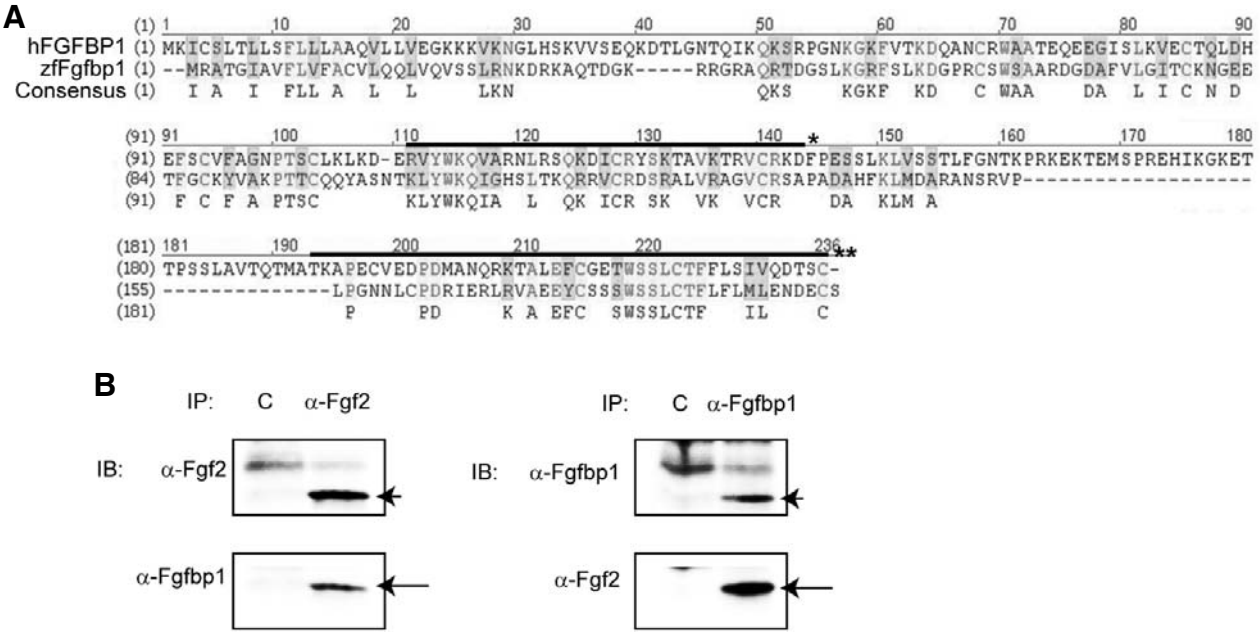


Fig. 1. Zebrafish Fgfbp1 shares sequence similarity and function with human FGFBP1. (A) Amino acid sequences were aligned for human and zebrafish FGFBP1 using vector NTI (invitrogen). Domains for heparin binding (*) and FGF2 binding (**) are underlined. (B) Association of zebrafish Fgfbp1 and Fgf2 is demonstrated by co-IP using 16 hpf zebrafish lysates. For the control IP, mouse pre-immune serum was used. Arrowheads indicate IP bands and arrows indicate co-IP bands.

mMachine kit (Ambion, USA) and co-injected with the MO.

In situ hybridization

In situ RNA hybridization was performed as previously described (Hauptmann and Gerster, 1994). Sense and antisense probes were obtained using a DIG RNA labeling kit (Roche, Germany) with SP6 or T7 RNA polymerases from *fgfbp1* or *fgf2* cloned in *pCR-Blunt II-TOPO* (Invitrogen, USA). The zebrafish *fgf2* clone contained an extra 100 bp of 5′ UTR sequences. Following color development, embryos were mounted on microscope slides in 75% glycerol. Images were obtained using a QImaging Retiga Exi color CCD camera mounted on a Zeiss Axioplan2 (Carl Zeiss, Germany).

Antibody (Ab) generation, immunoprecipitation, and Western blotting

Mouse polyclonal antisera against GST-fused full length Fgfbp1 and Fgf2 were generated and tested on ectopic proteins expressed in 293T cells. For immunoprecipitation (IP), zebrafish embryos (16 hpf) homogenized in NETN buffer (150 mM NaCl, 20 mM Tris-Cl, pH 8.0, 0.5% v/v Nonidet P-40, 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin A, 2 μg/ml Na3VO4, and 1 μg/ml leupeptin) were incubated overnight with the polyclonal antisera and protein G beads. For Western blotting, five embryos were lysed in Lamaeli buffer at the indicated time points. Whole lysates were loaded onto 15% polyacrylamide gels and transferred to the nitrocellulose paper. After blocking in 5% skim milk in TBS-0.1% Tween 20, blots were incubated with each antiserum or anti-α/β tubulin Ab (Cell Signaling, USA). Blots were probed with HRP conjugated anti-mouse or anti-rabbit Ab (Invitrogen), and signals detected with chemiluminescence.

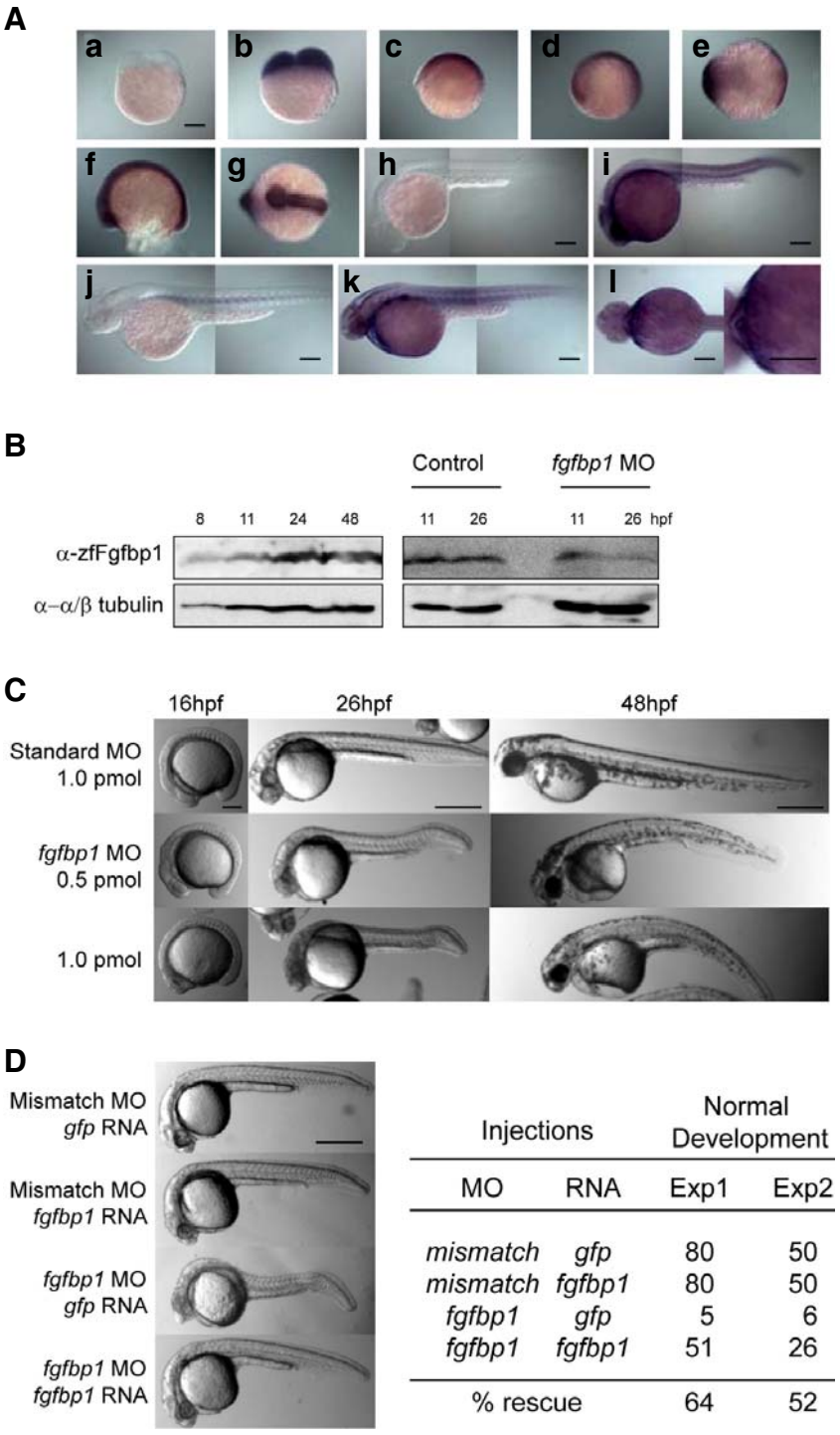
TUNEL staining and immunohistochemistry

Embryos were fixed in 4% paraformaldehyde and dehydrated in methanol. After rehydration, TUNEL staining was performed using an *in situ* cell death detection kit (Roche) according to the manufacturer's instructions. Antibody staining was performed using anti-pH3 Ab (Millipore, USA), anti-pERK Ab (Cone E10, Cell Signaling) or pAKT Ab (Clone587F11, Cell signaling). Phospho-H3 was detected by Alexa568-conjugated anti-mouse Ab (Invitrogen) and stained embryos were mounted on slides using vectashield mounting medium with DAPI (Vector Lab., USA). Phospho-ERK and pAKT were detected by biotinylated anti-mouse Ig Ab and Avidin/HRP using DAB (Vector Lab) as a color substrate. Fluorescence images were captured using a Zeiss Lumar Axioscope and color images with Zeiss Axioplan2 (Carl Zeiss).

RESULTS

FGFBP1 is ubiquitously expressed during zebrafish embryogenesis

To study the role of *fgfbp1* in zebrafish, we cloned the putative zebrafish *fgfbp1* gene (XM_001332777) from 24 hpf embryos. Sequence alignment between human and zebrafish FGFBP1 shows an overall 24% amino acid identity, with 41% identity (67% positive match) for the heparin binding domain and 38% identity (61% positive match) for the FGF binding domain (Fig. 1A). Zebrafish Fgfbp1 protein was able to bind zebrafish Fgf2 (Fig. 1B), suggesting that the function of FGFBP1 is conserved between humans and zebrafish. Zebrafish *fgfbp1* mRNAs are expressed from early stage embryos as maternal transcripts and actively transcribed throughout embryogenesis (Fig. 2A). At 24 hpf, high levels of mRNA expression can be detected in the eye, brain, heart, trunk, and yolk areas. Fgfbp1 protein was also detected by mouse polyclonal antisera generated against zebrafish Fgfbp1 (Fig. 2B). To investigate the developmental role of *fgfbp1*, we injected translation blocking MO into 1-4 cell stage embryos. Morphants receiving 0.5-1.0 pmol MO showed growth retardation and cell death, prominently shown as dark



Injections		Normal Development	
MO	RNA	Exp1	Exp2
mismatch	*gfp*	80	50
mismatch	*fgfbp1*	80	50
fgfbp1	*gfp*	5	6
fgfbp1	*fgfbp1*	51	26
% rescue		64	52

Fig. 2. Zebrafish *fgfbp1* is ubiquitously expressed and essential for development. (A) Spatio-temporal expression of *fgfbp1* mRNA was assessed by *in situ* hybridization. Stained with sense probes are a, 2-cell; h, 24 hpf; j, 48 hpf. Stained with antisense probes are b, 2-cell; c, 70% epiboly; d and e, bud; f and g, 14 somites; i, 24 hpf; k and l, 48 hpf. Lateral views are presented except for e, g (animal pole at the center), and i (ventral view). Scale bar represents 20 μm. (B) Fgfbp1 protein expression was determined by western blotting. Knock-down after morpholino injection was determined. (C) Pictures were taken for the zebrafish embryos injected with standard or *fgfbp1* MOs. Morphants show prominent cell death and growth retardation. Injections were performed for 100 embryos for each group and repeated > 5 times with > 90% embryos showing similar phenotype. Scale bar represents 50 μm. (D) *fgfbp1* RNA rescues *fgfbp1* morphants from developmental abnormalities and death. Representative pictures were taken at 24 hpf for the zebrafish embryos injected with 1.0 pmol of *fgfbp1* MO or a mismatch MO in combination with 250 pg of *gfp* or *fgfbp1* RNA. Scale bar represents 50 μm.

areas at the head region (Fig. 2C, 26 hpf). At 48 hpf, surviving morphants lost the refringeness in the trunk and showed pericardial sac enlargement. Most embryos were extremely fragile and died around 4-5 days post fertilization. We tested the specificity of the *fgfbp1* MO by measuring the protein knock-down and performing rescue experiments. The knock-down efficiency was determined for the embryos injected with 1.0 pmol MO (Fig. 2B), whose Fgfbp1 protein expression was diminished by 70% at 26 hpf. The rescue experiments were performed with 1.0 pmol MO and 250 pg of synthetic RNA. As shown in Fig. 2D, ectopic *fgfbp1* RNA rescued more than 50% of the morphants, demonstrating the specificity of the *fgfbp1* MO. We only counted normal looking embryos alive at 5 dpf and excluded partially rescued embryos for the measurement. Taken together, our data demonstrate that the *fgfbp1* is ubiquitously expressed and essential for zebrafish embryogenesis.

FGFBP1 is required for cellular survival throughout zebrafish embryogenesis

Phenotypic analyses of *fgfbp1* morphants suggested that cell

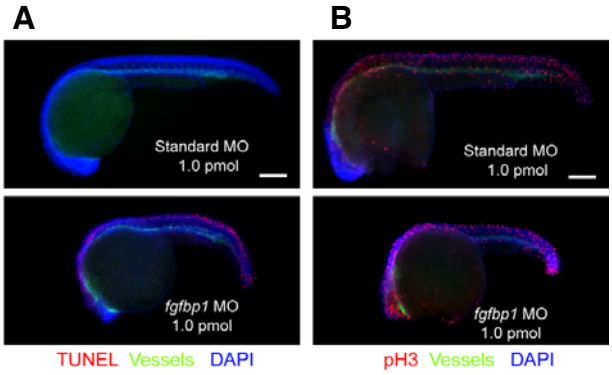


Fig. 3. Zebrafish *fgfbp1* knock-down induces apoptosis. Standard or *fgfbp1* MOs were injected into fli1a:egfp transgenic embryos. At 24 hpf, TUNEL (A) or pH3 (B) staining was performed for embryos injected with standard MO or 1.0 pmol *fgfbp1* MO to assess apoptosis and cell proliferation respectively. TUNEL$^+$ cells were detected with TMR red (A) and pH3$^+$ cells with Alexa568 (B). GFP (green) marks the vessels and DAPI (blue) lines for the zebrafish mass. Scale bar represents 20 μm.

death was induced after the knock-down. Therefore, we investigated whether the cells in the morphants underwent apoptosis using TUNEL staining. As shown in Fig. 3A, extensive apoptosis was observed at the inner cell mass in the head and trunk regions. Cell death was most prominent for the brain and the cells forming the neural tube but not restricted to those regions. The apoptotic regions are identical or close to the areas where *fgfbp1* mRNA is highly expressed, suggesting that Fgfbp1 exerts pro-survival function in an auto- or paracrine manner. Because the morphants manifested severe growth retardation, we also assessed phosphohistone H3 (pH3) expression to score the number of mitotic cells (Fig. 3B). In contrast to the TUNEL staining results, levels of pH3 staining were not markedly affected by *fgfbp1* knock-down. These results indicate that the primary function of embryonic *fgfbp1* is to prevent cell death, thus ensuring cellular survival rather than promoting cellular proliferation.

In mouse tumor xenograft studies and chicken chorioallantoic assays, FGFBP1 was shown to promote angiogenesis (Czubayko et al., 1994; Tassi et al., 2001). Therefore, we investigated whether *fgfbp1* knock-down affects vessel formation by utilizing a (fli1a:egfp) transgenic zebrafish (Lawson and Weinstein, 2002). This transgenic line expresses GFP in the vascular endothelial cells, enabling easy assessment of vasculogenesis. As shown in Figs. 4B and 4E, vasculogenesis was not disturbed by 0.25 pmol *fgfbp1* MO injection, which induced a slightly bent trunk. With increasing amounts of MO injection (0.5-1.0 pmol), embryos were severely deformed and vessels were underdeveloped (Figs. 4C and 4F). At 72 hpf, surviving embryos fully developed inter-segmental vessels, yet failed to form sub-intestinal vessels (SIV, I and J). At this point, pericardial edema and pericardial sac enlargement were also observed (Fig. 2C). These data clearly implicate a role of Fgfbp1 in normal vasculogenesis, yet accompanying developmental abnormalities raise a possibility of indirect effects due to cell death in the neighboring regions. Taken together, our results demonstrate that the primary function of Fgfbp1 is to sustain cellular survival throughout zebrafish embryogenesis, and its depletion causes abnormal formation of multiple structures including vessels and the heart.

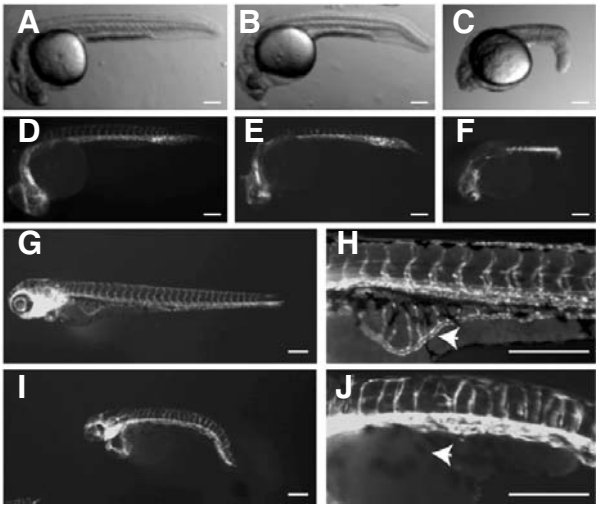


Fig. 4. Zebrafish *fgfbp1* knock-down induces defects in the vasculogenesis. Standard or *fgfbp1* MOs were injected into fli1a:egfp transgenic embryos. Vessel formation is visualized by GFP signal. Compared to the standard MO injected embryos (A, D), *fgfbp1* morphants given 1.0 pmol *fgfbp1* MO show underdeveloped vessels (C, F) at 24 hpf. At 72 hpf, morphants given 1.0 pmol *fgfbp1* MO failed to grow subintestinal vessels (arrowheads, compare I and J with control G and H). Scale bar represents 20 μm.

FGFBP1 activates FGFR signaling cascade

Previous studies suggested that the function of FGFBP1 was largely attributed to FGF2 mobilization and activation of FGFR signaling. Therefore we assessed whether zebrafish Fgfbp1 triggered FGFR downstream signaling. Activation of FGFR by basic FGFs elicits two major signaling pathways, i.e., activation of Ras-MAPK and PI-3 kinase/AKT cascade (Eswarakumar et al., 2005; Korc and Friesel, 2009). In order to assess MAPK signaling, we stained zebrafish embryos with pERK and found widespread expression in the eye, brain, and the trunk at 24 hpf (Fig. 5A). In contrast, pERK expression was almost undetectable in the *fgfbp1* morphants, suggesting a major involvement of Fgfbp1 in MAPK signaling. For the assessment of PI-3/AKT signaling activation, we examined phosphorylation of AKT at Ser473. Compared to the strong expression of pAKT in the standard MO injected embryos, *fgfbp1* morphants had reduced levels of pAKT expression, manifesting the attenuation of PI-3/AKT signaling. Together, these data suggest that zebrafish Fgfbp1 indeed activates FGFR and the *fgfbp1* morphant phenotypes are due to the abrogation of FGFR signaling.

FGF2 plays an anti-apoptotic function at an early stage of embryogenesis

Next, we assessed the expression of *fgf2* and its knock-down phenotype in zebrafish. Zebrafish *fgf2* transcripts are maternally expressed, and their levels were elevated after zygotic mRNA expression (Figs. 6Ab and 6Ac). At 24 hpf, mRNA expression was reduced and restricted to the eyes and the brain, and no longer detectable at 48 hpf, indicating that *fgf2* is transcribed when cells are undergoing vigorous proliferation. Protein expression showed a similar pattern, with levels peaking at 16 hpf (Fig. 6B). In the knock-down experiments, zebrafish receiving 0.1-0.5 pmol *fgf2* translation blocking MO underwent massive apoptosis in the brain (Fig. 6C) and failed to survive beyond the 14-somite stage. Therefore the pro-survival function of Fgf2 was most critical during early embryogenesis. This phenotype

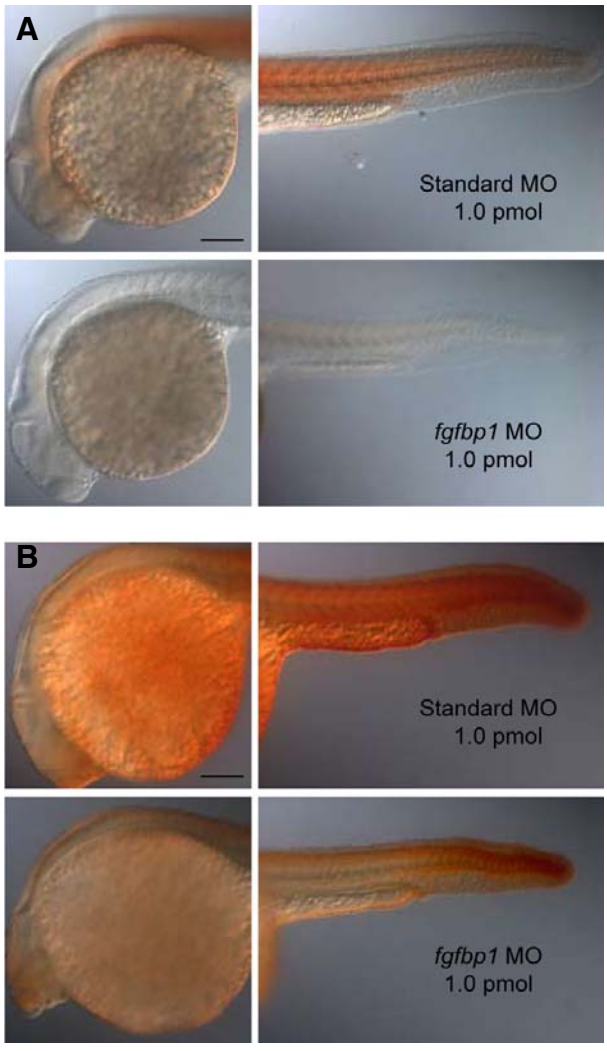


Fig. 5. Zebrafish *fgfbp1* knock-down reduces pERK and pAKT expression. One pmol of standard or *fgfbp1* MOs were injected into 1-4 cell embryos and IHC was performed at 24 hpf. (A) Standard embryos showed widespread pERK expression whereas *fgfbp1* morphants completely lacked pERK. All embryos (n = 40) had no pERK staining. (B) Standard embryos showed widespread pAKT expression whereas *fgfbp1* morphants had reduced levels of pAKT. Most embryos had reduced pAKT signal (n = 38 out of 40). Scale bar represents 20 μm.

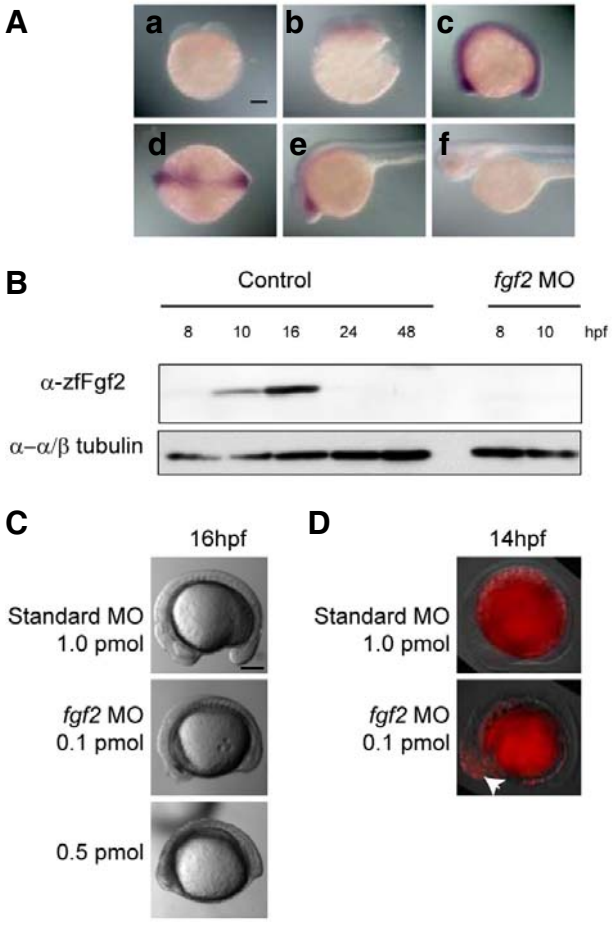


Fig. 6. Zebrafish Fgf2 is essential for cellular survival during early embryogenesis. (A) Spatio-temporal expression of *fgf2* mRNA was assessed by *in situ* hybridization. *fgf2* mRNAs are present as maternal transcripts (b, 2-cell) and the expression is maintained until 14 somites (c and d). At 24 hpf, mRNA is only detected in the eyes and the brain (e) and disappeared at 48 hpf (f). Sense probe was used for a 2-cell embryo for (a). Lateral views are shown and the scale bar represents 20 μm. (B) Fgf2 protein expression and knock-down efficiency was determined by western blotting. (C) Standard or *fgf2* MOs were injected into 1-4 cell stage zebrafish embryos. Embryos given more than 0.5 pmol MO collapsed before 14 somites. Scale bar represents 20 μm. (D) Embryos given 0.1 pmol *fgf2* MO were subjected to the TUNEL staining to assess cell death. TUNEL positive cells are concentrated in the head region (arrowhead).

resembles the cerebral cortical abnormalities of *Fgf2* knock out mice, but differs from mice as *Fgf2*-deficient mice were viable and showed no increase in cell death (Dono et al., 1998; Ortega et al., 1998; Vaccarino et al., 1999). Collectively, our data show that both Fgf2 and Fgfbp1 are required for the cellular survival during zebrafish embryogenesis, yet the difference in the timing of their expression suggests that Fgfbp1 could work independently from Fgf2.

DISCUSSION

In this study, we found that zebrafish *fgfbp1* is highly expressed throughout embryogenesis and is essential for cellular survival. As previously suggested, the function of FGFBP1 may be to regulate the biological activities of FGF2 (Presta et al., 2005; Wu et al., 1991). Indeed, zebrafish Fgfbp1 activated the signaling pathways typically associated with the basic FGFs. However, our data also indicate that Fgfbp1 plays a pro-survival function in the absence of Fgf2 at later embryonic stages. In the absence of FGF2, FGFBP1 may pair with other FGF family members, such as FGF1, 7, and/or 10 (Beer et al., 2005). Although this association needs to be confirmed, the Fgf2 independence suggests that human FGFBP1 could be a promising therapeutic target for the modulation of basic FGF-FGFR signaling in clinical settings. In cardiovascular diseases, FGF2 has been proposed as a target for therapeutic angiogenesis, yet clinical trials showed only limited efficacy (Aviles et al., 2003). Several factors may have affected these results, such as low therapeutic dose due to side effects and redundant actions of

other FGF family members. Because our data suggest that FGFBP1, an important regulator of FGF2, could exert biological activities independent of FGF2 and over a broad timeline, it will be worthwhile to consider FGFBP1 for therapeutic angiogenesis. Similarly, FGFBP1 may be a good target for anti-angiogenic therapy in cancer where FGF2 and FGFBP1 synergistically induce vessel formation.

To assess whether Fgfbp1 affects the FGFR signaling cascade, we looked at pERK as a marker for the MAPK pathway, and pAKT, which indicates PI-3 kinase/AKT pathway activation. Although these two pathways are known to be the major pathways downstream of FGFR, other growth factor-receptors also trigger activation of these pathways. Thus, it was rather surprising to see the complete absence of pERK expression in *fgfbp1* morphants. This may be due to the requirement of FGFR signaling for the induction of other factors or for the survival of cell populations producing those factors. By comparison, pAKT expression was decreased but present throughout the body of zebrafish embryos, indicating that the role of FGFBP1 in the activation of the PI-3 kinase/AKT pathway is limited. In fact, VEGFR signaling was shown to be responsible for pAKT expression and cellular survival in zebrafish endothelial cells (Chan et al., 2002). The dramatic effect of *fgfbp1* MO on pErk expression suggests that the anti-apoptotic function of FGFBP1 is likely due to the activation of the MAPK pathway. The pro-survival function of MAPK activation has been demonstrated in many experimental systems (Grant et al., 2002; Panka et al., 2006), and the inhibition of MAPK has been shown to cause de-phosphorylation and accumulation of pro-apoptotic proteins, such as Bad and Bim (Eisenmann et al., 2003; Harada et al., 2004; Ley et al., 2003). Thus, it will be interesting to see if these pro-apoptotic molecules also accumulate in *fgfbp1* morphants.

Collectively, our results show that zebrafish Fgfbp1 is absolutely required for the activation of MAPK signaling and cellular survival, and highlight zebrafish as a good model for dissecting the role of extracellular matrix proteins.

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