



Published in final edited form as:

*J Cell Physiol.* 2011 July ; 226(7): 1722–1726. doi:10.1002/jcp.22587.

## Numerous isoforms of *Fgf8* reflect its multiple roles in the developing brain

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### Abstract

Soluble growth factors play an important role in the coordination and integration of cell proliferation, differentiation, fate determination and morphogenesis during development of multicellular organisms. Fibroblast Growth Factors (FGFs) are a large family of polypeptide growth factors that are present in organisms ranging from nematodes to humans. RNA alternative splicing of FGFs and their receptors further enhances the complexity of this ligand-receptor system. The mouse *Fgf8* gene produces eight splice variants, which encode isoform proteins with different N-termini and distinct receptor binding affinity and biological activity. In this article, we review the roles of *Fgf8* in vertebrate development and summarize the recent findings on the in vivo function of different *Fgf8* splice variants. We propose that multiple *Fgf8* isoform proteins act in concert to regulate the overall function of *Fgf8* and account for the diverse and essential role of *Fgf8* during vertebrate development.

### The FGF family in vertebrates

The first two FGFs, Fgf1 and Fgf2, were isolated as mitogens for fibroblast from bovine pituitary and brain (Gospodarowicz, 1975; Gospodarowicz et al., 1978; Gospodarowicz et al., 1974; Maciag et al., 1979). Subsequent studies have identified numerous FGFs in various organisms, except for unicellular organisms (Itoh and Ornitz, 2008). In mice and humans, the FGF family is comprised of 22 members, which are grouped into six or seven subfamilies based on similarity in sequence, gene location and function. Genome sequence analyses suggest that the FGF family may have expanded in two phases by gene duplication during evolution (Itoh and Ornitz, 2004; Itoh and Ornitz, 2008). The first phase was associated with early metazoan evolution to generate ancestors of 6–7 FGF subfamilies. Subsequently, large-scale genome duplication during the early evolution of vertebrates further expanded the FGF subfamilies to contain 3–4 members.

Most of FGF members are secreted proteins with a clear N-terminal signal peptide. FGFs 1, 2, 9, 16, and 20 are secreted proteins though they lack an obvious cleavable N-terminal signal sequence. FGFs 11–14 are intracellular proteins and thus called intracellular FGF (iFGF) (Itoh and Ornitz, 2008). All FGFs contain a conserved core of approximately 120 amino acids arranged into a globular domain composed of 12 antiparallel  $\beta$  strands (Eriksson et al., 1991; Zhu et al., 1991). This core domain is important for receptor binding, while the divergent N- and C-termini of FGF can modify the receptor binding affinity and account for the different biological function of FGF (Mohammadi et al., 2005). In humans and mice, there are four FGF receptors, FGFR1–4, which are receptor tyrosine kinases and contain an extracellular ligand-binding domain with three immunoglobulin domains (I, II,

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and III), a single-pass transmembrane domain, and intracellular tyrosine kinase domain (Mohammadi et al., 2005; Ornitz and Itoh, 2001). Alternative splicing of FGFR1-3 genes produces two different isoforms with different immunoglobulin domain III (IIIb and IIIc), which display distinct tissue-specific expression pattern and ligand-binding affinity (Mohammadi et al., 2005). These four FGFRs mediate the function of all FGFs, except for iFGFs (Goldfarb, 2005; Olsen et al., 2003).

FGFs have binding sites for heparin and heparan sulfate, which concomitantly interact with FGF and FGFR and thereby facilitate the formation of a ternary FGF-FGFR-heparin complex (Mohammadi et al., 2005; Nugent and Edelman, 1992; Schlessinger et al., 2000). Therefore, heparin sulfate is considered a co-receptor of FGF. Because of their high affinity of binding with FGFs and the abundance of heparan sulfate proteoglycans on cell surface and in extracellular matrix, FGFs are mostly retained near the site of production (Itoh and Ornitz, 2008). However, FGFs 19, 21, 23 have low-affinity heparin binding sites and thus act in an endocrine manner (Goetz et al., 2007). The low heparin-binding affinity results in the dependence of these three FGFs on Klotho/ $\beta$ Klotho as their co-receptor to act only in Klotho-expressing tissues (Goetz et al., 2007).

## Expression and diverse function of FGF8 during vertebrate development

FGF8 was originally identified as an androgen-induced growth factor in a mouse mammary tumor cell line, SC-3 cells (Tanaka et al., 1992). Subsequent studies have revealed its important role in different developmental processes, including cell specification, differentiation, proliferation, survival, and transformation. During mouse embryogenesis, *Fgf8* is expressed at sites that are known to instruct growth and patterning, such as the primitive streak and tail bud, the apical ectodermal ridge (AER) of the limb bud, and the midbrain-hindbrain junction, anterior neural ridge (Crossley and Martin, 1995; MacArthur et al., 1995b). In addition, *Fgf8* expression is found in branchial arches, the hypothalamus, and otic vesicles (Crossley and Martin, 1995; Crossley et al., 2001).

Genetic studies have demonstrated that *Fgf8* plays an important role during embryogenesis in mice. Before and during gastrulation, *Fgf8* is essential for morphogenetic remodeling of embryos by promoting formation and migration of the mesoderm (Guo and Li, 2007; Sun et al., 1999). Toward the end of gastrulation, the persistent *Fgf8* expression in the tailbud and the posterior end of the neural tube patterns the neural tube and paraxial mesoderm (Diez del Corral et al., 2003; Dubrulle and Pourquie, 2004). Furthermore, the expression of *Fgf8* in the primitive streak is essential for development of the left-right asymmetry of the internal organs (Meyers and Martin, 1999). Because of the critical role of *Fgf8* during early embryogenesis, mouse embryos homozygous for *Fgf8*-null mutation die at embryonic day (E) 8.5 (Sun et al., 1999). Generation of hypomorphic mutation of *Fgf8* and floxed conditional mutation, which allows tissue-specific knockout experiments using the *Cre-LoxP* system, has been instrumental in investigating the function of *Fgf8* in the development of multiple tissue/organ systems after E8.5 (Meyers et al., 1998). Remarkably, these experiments have revealed that *Fgf8* plays a crucial role in most of the tissues or organs in which *Fgf8* transcripts are present. For example, deletion of *Fgf8* results in malformation of major brain structures (Garel et al., 2003; Martinez-Ferre and Martinez, 2009; Storm et al., 2003), abnormal development of olfactory neurogenesis and the nasal cavity (Kawauchi et al., 2005), cardiovascular and craniofacial structures (Frank et al., 2002; Trumpp et al., 1999), limbs (Lewandoski et al., 2000; Moon and Capecchi, 2000), inner ear (Ladher et al., 2005), and kidneys (Perantoni et al., 2005). In humans, mutations in the FGF8 genes have been implicated in congenital malformations and pathological conditions in adults. *FGF8* mutations has been associated with non-syndromic cleft lip and/or palate (Riley et al., 2007). Furthermore, mutations of FGF8 cause idiopathic hypogonadotropic hypogonadism (IHH,

also termed congenital gonadotropin-releasing hormone deficiency) (Falardeau et al., 2008; Trarbach et al., 2010).

## Alternative splicing generates different FGF8 isoforms with different biological activities and function

Alternative splicing is known to be common in higher eukaryotic organisms. In humans, more than 98% of multi-exonic genes in the genome undergo alternative splicing, generating multiple splice variants and greatly augmenting genetic and functional complexity without a concomitant increase in the genome size (Pan et al., 2008; Wang et al., 2008). The *FGF8* gene produces one of the most complex transcript variants through alternative splicing among the FGF family.

Most *FGF* genes contain a 3-exon genomic structure (Ornitz and Itoh, 2001). The first exon of the *FGF8* gene is further subdivided into four alternatively spliced sub-exons in human and mouse (Crossley and Martin, 1995; Gemel et al., 1996; MacArthur et al., 1995b) (Fig. 1A). In mice, the *Fgf8* splice variants produce eight potential isoform proteins, Fgf8 a–h, of different N-termini (Blunt et al., 1997; Crossley and Martin, 1995; MacArthur et al., 1995b). In humans, a stop codon in *FGF8* exon 1B limits the number of isoforms to four, corresponding to mouse Fgf8 a, b, e and f isoforms (Gemel et al., 1996; Ghosh et al., 1996). Interestingly, *FGF8a* and *FGF8b* have 100% sequence homology between mouse and man, while *FGF8* as a whole has 98% homology (Ghosh et al., 1996). Comparison of the genomic sequence of *FGF8* genes in different species shows that, although the last three exons (1D, 2, and 3) are highly conserved, the upstream exons are extremely divergent (Fig. 1B). Exon 1C is absent in zebrafish, *Xenopus* and chicken, and only *Fgf8a* and *Fgf8b* have been identified in these organisms (Haworth et al., 2005; Inoue et al., 2006; Shim et al., 2005). The exon 1C also appears absent in opossum (Fig. 1B). Therefore, the acquisition of isoforms e, f, g, and h is probably recent in evolution and these FGF8 isoforms are only present in placental mammals.

## Expression of different Fgf8 splice variants

Alternative splicing is often regulated, and switching production of spliceforms encoding proteins of different activity plays an important regulatory role during development. However, there is currently no evidence that the alternative splicing of *FGF8* are regulated, at least during vertebrate embryogenesis. Expression studies of *Fgf8* splice variants in zebrafish, *Xenopus*, and chick have shown that both *Fgf8a* and *Fgf8b* are co-expressed, with *Fgf8b* being the predominant spliceform (Inoue et al., 2006; Sato et al., 2001; Shim et al., 2005). In mice, at least seven *Fgf8* splice variants were detected in various *Fgf8* expressing tissues (Blunt et al., 1997; MacArthur et al., 1995b). RT-PCR using a single pair of primers that detect all *Fgf8* spliceforms indicated that the relative level of different splice variants remains constant in different tissues and at different stages (Blunt et al., 1997)(Li et al, unpublished data). Although Brondani and colleagues showed that retinoic acid induces switch of *FGF8b* to *FGF8a* in the prostate cancer cell line LNCaP (Brondani and Hamy, 2000; Brondani et al., 2002), treatment with retinoic acid does not affect the relative levels of *fgf8a* and *fgf8b* transcripts in *Xenopus* gastrula explants or *Xenopus* embryos *in vivo* from the early gastrula to the neurula stage (Shim et al., 2005). Therefore, the overall function of *FGF8* is likely controlled by the concerted action of different isoforms, depending on the availability of receptors, co-receptors, and other components of FGF signaling in the specific cellular context.

## FGF8a and FGF8b have distinct activities and different binding affinities with FGF receptor

Numerous studies using different assay systems have demonstrated that two of the evolutionarily conserved FGF8 isoforms, FGF8a and FGF8b, have different biological activities. It was first reported that FGF8b had much greater transforming activity on NIH3T3 cells than FGF8a, and cells transfected with *FGF8b* were more tumorigenic (MacArthur et al., 1995a). Subsequently, the distinct activity of Fgf8a and Fgf8b was demonstrated by misexpression experiments in the developing midbrain and hindbrain in the mouse and chick embryo. *Fgf8* is normally expressed at the midbrain-hindbrain junction, called the isthmus (Crossley and Martin, 1995). Transplantation experiments have revealed that grafts of the isthmus tissue can induce ectopic midbrain or cerebellum in competent regions of the neural tube (Alvarado-Mallart, 1993; Le Douarin, 1993). Recombinant Fgf8b protein possesses the similar inductive activity of the isthmus tissue (Crossley et al., 1996). Subsequent studies using transgenic mice or *in ovo* electroporation in chick embryos show that *Fgf8a* and *Fgf8b* have distinct activities in the developing midbrain. Misexpression of *Fgf8a* causes massive proliferation of midbrain cells and transformation of the posterior forebrain into a midbrain fate (Lee et al., 1997; Liu et al., 1999; Sato et al., 2001). On the other hand, misexpression of *Fgf8b* transforms midbrain cells to a hindbrain fate (Liu et al., 1999). Experiments in zebrafish and *Xenopus* embryos have also demonstrated different activity between fgf8a and fgf8b (Fletcher et al., 2006; Inoue et al., 2006; Shim et al., 2005).

Mitogenic studies using transfected BaF3 cell lines expressing different FGFRs have indicated that different FGF8 isoforms have distinct binding specificities with different FGFRs and their isoforms, suggesting the divergent function of FGF8a and FGF8b may result from their selective binding with FGFRs (MacArthur et al., 1995b). Given the remarkable divergence in their activity, the only difference between FGF8a and FGF8b is the presence of an additional 11 amino acids in the N-terminus of FGF8b. It was speculated that an N-glycosylation site, NFTQ, within these 11 amino acid residues might contribute to the unique function of FGF8b (Crossley and Martin, 1995; Shim et al., 2005). Indeed, glycosylation has been shown to modulate receptor binding affinity and the activity of other FGFs (Asada et al., 1999; Bellosta et al., 1993; Duraisamy et al., 2001). However, we found that mutating N31 to alanine did not affect the activity of Fgf8b, demonstrating that N-glycosylation is probably not essential for the Fgf8b-specific activity (unpublished data). Structural analysis shows that F32 mediates an additional contact with Ig domain III of the receptors FGFR1c, 2c, 3c and FGFR4, and this additional contact results in a significant increase in the binding affinity with FGFRs (Olsen et al., 2006). Moreover, substitution of F32 to alanine renders FGF8b<sup>F32A</sup> to have a similar binding affinity with FGFR1c, 2c, 3c and FGFR4 as FGF8a; forced expression of FGF8b<sup>F32A</sup> causes the same phenotype as that of FGF8a in the embryonic midbrain of chick embryos (Olsen et al., 2006). Therefore, the increased binding affinity of FGF8b with FGFRs accounts for the FGF8b-specific activity.

It remains to be determined whether the activity of Fgf8a and Fgf8b differs in the intensity or the pathway of FGF8 signaling. Electroporation of reduced concentration of *Fgf8b* produces the same phenotype as *Fgf8a* in the chick embryo (Sato et al., 2001). However, electroporation of increased concentration of *Fgf8a* in chick embryos (unpublished data), or injection of 100x more *Fgf8a* RNA does not produce the same phenotype as *Fgf8b* in *Xenopus*, suggesting that there is quality difference in the signaling generated by Fgf8a or Fgf8b (Fletcher et al., 2006). In support of this notion, only *Fgf8b* induces the Ras-ERK signaling pathway in chick midbrain (Sato and Nakamura, 2004). Differences in the kinetics of ligand-receptor interaction may modulate the quality of signal transduction. Interestingly, structural analysis suggests that FGF8b binding induces rotation and closer juxtaposition of

cytoplasmic kinase domains of FGF-FGFR dimers, which may ultimately change the kinetics and quality of signal transduction (Olsen et al., 2006).

### In vivo requirement for Fgf8a and Fgf8b isoforms during development

Loss-of-function experiments have identified the relative contribution of *FGF8a* and *FGF8b* to the overall *FGF8* function during embryogenesis. Experiments in *Xenopus* using morpholino oligonucleotides to eliminate *fgf8a* and *fgf8b* together or *fgf8a* alone demonstrate that *fgf8b*, but not *fgf8a*, is required for proper gastrulation and mesoderm formation, whereas deletion of *fgf8a* causes a reduction in the specification of posterior neural tissues (Fletcher et al., 2006). Mice carrying a point mutation at the splice acceptor that abolishes *Fgf8b*-containing spliceforms (b, d, f, and h) display an identical defect in morphogenetic remodeling as embryos homozygous for *Fgf8*-null mutation before gastrulation (Guo and Li, 2007). However, the remaining *Fgf8a*-containing spliceforms (a, c, e, and g) can partially compensate for the loss of *Fgf8b*-containing spliceforms in mesoderm specification and migration, both of which are severely disrupted in *Fgf8*-null embryos, during gastrulation (Guo and Li, 2007; Sun et al., 1999). In contrast to their role in gastrulation, *Fgf8b*-containing spliceforms are absolutely essential for *Fgf8* function in the formation of the midbrain and hindbrain (MHB) (Guo et al., 2010). Deletion of *Fgf8b*-containing spliceforms leads to loss of multiple key regulatory genes, including *Fgf8* itself, in the isthmus. Therefore, specific inactivation of *Fgf8b*-containing spliceforms, similar to the complete deletion of *Fgf8*, in MHB progenitors results in loss of the midbrain, isthmus, and cerebellum (Chi et al., 2003; Guo et al., 2010). Conversely, deletion of *Fgf8a*-containing isoforms has no discernable effects on MHB development (Guo et al., 2010). Interestingly, mice lacking *Fgf8a*-containing spliceforms display phenotypes, such as growth retardation, reduced fertility, and partial postnatal lethality, and the severity of the phenotype is dependent on genetic backgrounds (Guo et al., 2010). These results suggest that, although *Fgf8b*-containing isoforms have a dominant function, *Fgf8a*-containing spliceforms may play a subtle role in fine-tuning *Fgf8* function in tissues outside the MHB region.

### Other FGF8 isoforms may also contribute to the overall FGF8 function

There is evident that, in addition to FGF8a and Fgf8b, other FGF8 isoforms may also contribute to the overall FGF8 function. Experiments using BaF3 cells expressing different FGFRs have revealed that recombinant Fgf8c and Fgf8f have a strong mitogenic activity by activating Fgfr2c, Fgfr3c and Fgfr4 (Blunt et al., 1997; MacArthur et al., 1995b). Mouse *Fgf8f* exhibits similar potency as human and mouse *FGF8b* in inducing mesoderm in *Xenopus* embryo explants and expanding the mesodermal domain in *Xenopus* embryos (Fletcher et al., 2006). Given that FGF8f contains the N-terminal sequence that is crucial for FGF8b activity, it may not be surprising that FGF8f has similar activity as FGF8b (Falardeau et al., 2008; Olsen et al., 2006). In support of the potential function FGF8f, both FGF8b and FGF8f are induced by androgens in squamous cell carcinoma line and the expression of FGF8f is detected and associated with progression of esophageal carcinomas (Tanaka et al., 2001). Furthermore, two loss-of-function mutations, P26L and F40L, that are located within exon 1C and thus affect only FGF8e and FGF8f isoforms have been identified in patients with idiopathic hypogonadotropic hypogonadism, which is associated with deficiency of gonadotropin-releasing hormone (GnRH) (Falardeau et al., 2008). Therefore, FGF8e and/or FGF8f isoforms may be important for the function of FGF8 in the formation GnRH neurons.

### Concluding remarks

Multiple FGF8 isoforms are co-expressed in various site of *FGF8* expressing cells. The function of FGF8 may, therefore, reflect the combined activity of a particular sum of FGF8

isoforms. As the addition of new Fgf8 isoforms occurs in placental mammals, Fgf8c–h may play distinct function from Fgf8a and Fgf8b, and probably contribute to the evolution of mammals. It is worth to note that several different FGF family members are often expressed in the same tissue during development. Interactions among different FGFs may produce activities different from each individual FGF. Therefore, experiments to dissect the relative contribution of different FGF8 isoforms to the overall FGF8 function can provide an excellent experimental paradigm to understand the interaction among FGFs in the control of vertebrate development. The remaining challenges are to determine the molecular bases underlying the different activity of FGF8 isoforms and the potential interaction among different FGF8 isoforms.

## Acknowledgments

We thank Dr. Jaime Rivera-Perez for critical reading of the manuscript. This work is supported by grants from the NIH (R01HD050474) and March of Dimes foundation to J. L.

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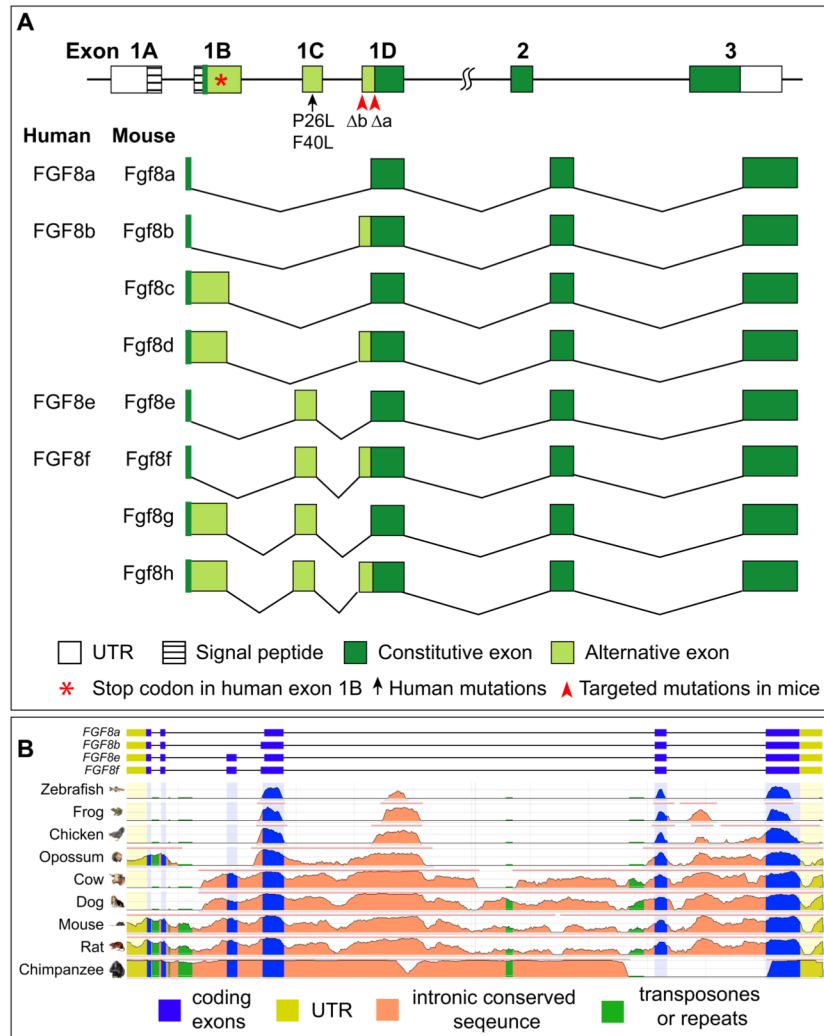
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**Figure 1.**

(A) Genomic structure and different splice variants of the *FGF8* gene in humans and mice. In humans, there is a stop codon (asterisk) in the exon 1B. Therefore, only four potential splice variants of human FGF8 instead of eight in mice. Targeted point mutations  $\Delta a$  and  $\Delta b$  (arrowheads) disrupt the splice acceptor in exon 1D abolishing *Fgf8a*-containing (*a*, *c*, *e*, and *g*) and *Fgf8b*-containing (*b*, *d*, *f*, and *h*) splice variants in mice. Missense mutations (arrow) have been identified in FGF8 exon 1C, which is only included in *FGF8e* and *FGF8f*, in patients with idiopathic hypogonadotropic hypogonadism. (B) Genome comparison of *Fgf8* genes in different species. Note that the first four exons are highly diverse while the last two exons are conserved. Exons 1C is missing in zebrafish, frog, chicken and opossum.