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Fibroblast growth factor receptors (FGFRs) and their roles in limb development

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Abstract Fibroblast growth factor (FGF) receptors constitute a family of four membrane-spanning tyrosine kinases (FGFR1–4) which serve as high-affinity receptors for 17 growth factors (FGF1–17). To study functions of FGF/FGFR signals in development, mice that carry mutations in each receptor have been created by gene targeting. Analysis of these mutant mice revealed essential functions of FGF receptors in multiple biological processes, including mesoderm induction and patterning, cell growth and migration, organ formation and bone growth. In this review we discuss recent work with FGF receptors to illustrate mechanisms, through which the FGF/FGFR signals specify vertebrate limb initiation, outgrowth and patterning.

Key words FGF-FGF receptors · Limb initiation · Progress zone activity · Hand and foot anomalies

Introduction

The developing limb proves to be an excellent model for studying interacting inductive signals. Limb development begins when cells from lateral plate mesoderm and nearby somites migrate to the presumptive limb field. The limb bud protrudes from the lateral body wall as a consequence of continued proliferation of mesenchymal cells at the appropriate axial levels at a time of reduced proliferation of cells in the rest of the flank (Summerbell et al. 1973). It is believed that inductive signals from proliferating mesodermal cells of the initiating limb bud induce the ectoderm at the tip of the bud to form a specialized structure called the apical ectodermal ridge (AER). An essential function of the AER in maintaining continuous limb bud outgrowth along the proximodistal (P-D, shoulder to digits) axis was first demonstrated 50 years ago by John W. Saunders. In that historical experiment, Saunders (1948) found that excision of the AER from a chick wing bud early in development generated wingless embryos. Removal of the AER at successively later stages of development resulted in the graded loss of more and more distal wing elements (Saunders 1948). Recently, it was found that the activities of the AER can be functionally replaced by ectopic application of fibroblast growth factors (FGFs) (Fallon et al. 1994; Laufer et al. 1994; Niswander et al. 1993). FGF signals originating from the AER are responsible for keeping the underlying mesenchyme (a region called the progress zone) in an undifferentiated, rapidly proliferating state (Dealy et al. 1997; Niswander and Martin 1993; Niswander et al. 1993).

Concomitant to its outgrowth along the P-D axis, the developing limb quickly establishes its anteroposterior (A-P, thumb to little finger) and dorsoventral (D-V, back of hand to palm) axes. It is known that sonic hedgehog (*Shh*), a signaling molecule produced in the zone of polarizing activity (ZPA), is involved in A-P patterning. *Shh* is normally expressed in the posterior mesenchyme. When cells expressing *Shh* or *Shh* protein itself were grafted to the anterior mesenchyme underneath the AER, they caused mirror-image duplication of digits (Chang et al. 1994; Lopez-Martinez et al. 1995; Riddle et al. 1993). Specification of the D-V axis seems to be determined by *En-1*, *Wnt7a* and *Lmx-1* (Loomis et al. 1998; Riddle et al. 1995; Vogel et al. 1995b). *Wnt7a* is expressed exclusively in the dorsal ectoderm and induces *Lmx-1* expression in dorsal mesenchyme (Riddle et al. 1995; Vogel et al. 1995). Loss of *Wnt7a* or *Lmx1* in mouse by gene targeting results in ventralization of the limb (Chen et al. 1998; Parr and McMahon 1995). Similarly, targeted disruption of *En-1* in mouse, which is expressed in ventral ectoderm, results in dorsalization of ventral mesoderm of limb bud (Loomis et al. 1996). Accumulating evidence indicated that signals from different functional centers of the three axes may interact with each other and coordinately regulate limb development and patterning (re-

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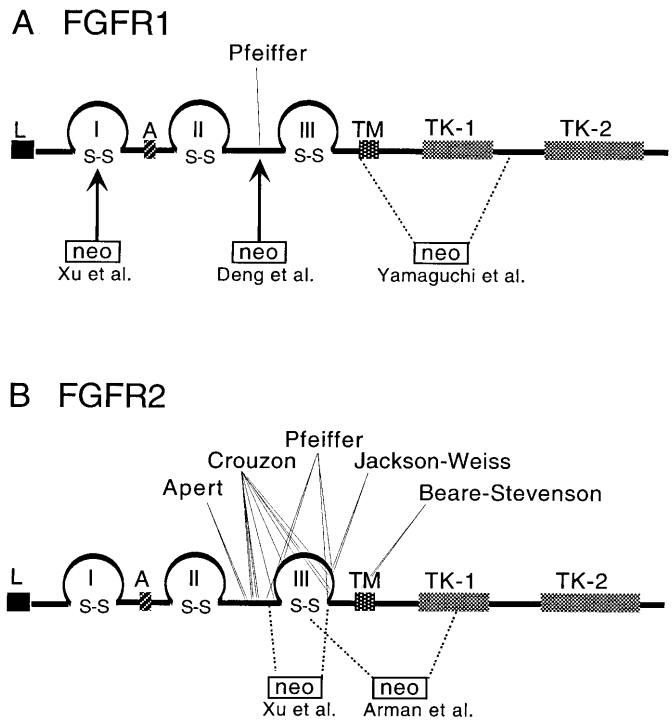

Fig. 1 Mutations of FGF receptor 1 (**A**) and receptor 2 (**B**). Targeted insertion of *neo* gene or deletion of endogenous sequences is indicated by *arrows* or *dotted lines*, respectively. The actual mutations that cause the diseases can be found in the following references: Addor et al. (1997); Lewanda et al. (1996); Muenke and Schell (1995); Nagase et al. (1998); Przylepa et al. (1996); Tartaglia et al. (1997); Wilkie et al. (1995)

viewed in Niswander 1996). A reciprocal activation loop between SHH of the ZPA and FGF4 of the AER is an excellent example of these sorts of interactions (Laufer et al. 1994; Niswander et al. 1994). Consistently, the loss of SHH in mouse by gene targeting not only affected the A-P axis, but also caused distal truncations along the P-D axis (Chiang et al. 1996). Many other signals are also found to be involved in limb development (reviewed in Johnson and Tabin 1997; Martin 1998; Tabin 1995; Tickle 1995). Here we try to avoid overlapping with these reviews and choose to concentrate mainly on the recent progress of FGF receptors in early development of the mouse limb bud. We will also briefly discuss FGF mutations and human limb abnormalities.

Fibroblast growth factors (FGFs) and their role in the early stages of limb development

FGFs are known to have an important role in limb initiation and patterning. The evidence is mainly derived from the following aspects. First, expression studies showed that at least five FGFs are expressed during limb bud initiation, with *Fgf2*, *Fgf4*, *Fgf8* and *Fgf9* expressed in the limb ectoderm and AER, and *Fgf2* and *Fgf10* expressed in the underlying mesenchyme (Crossley and Martin 1995; Crossley et al. 1996; Heikinheimo et al. 1994; Ohuchi et al. 1997; Savage and Fallon 1995; Vogel et al. 1996; Xu et al. 1998). Second, it was shown that FGF2 and FGF4 can substitute for AER signals and promote virtually complete outgrowth and patterning of the chick limb (Fallon et al. 1994; Niswander and Martin 1993; Niswander et al. 1993). Third, it was demonstrated that beads soaked with FGF1, FGF2, FGF4, FGF8 or FGF10 were capable of inducing the formation of complete, morphologically normal limb buds, when implanted in the presumptive flank of chick embryos (Cohn et al. 1995; Crossley et al. 1996; Ohuchi et al. 1997). Most importantly, a targeted disruption of the *Fgf10* gene in mouse resulted in mutant embryos without limbs (Min et al. 1998).

Despite all this evidence, interestingly, targeted disruption of at least eight FGFs in mice, including these ectoderm- and/or AER-located factors, failed to provide any information that linked them to limb induction. Although distinct developmental defects have been found in the mice that carry a null mutation of each FGF, the mutant mice either die at stages before limb initiation or survive to adulthood without any signs of limb abnormality (Table 1). Cre-loxP-mediated recombination has been used to eliminate FGF8 in the AER of embryonic mice at day 10.5 (E10.5). However, the mutant embryos could still initiate limb buds, although skeletal abnormalities were found in both forelimbs and hindlimbs, suggesting an involvement of other FGF genes in the limb induction (G. Martin, personal communication). Apparently, the analysis of the ligands for this critical issue is complicated by both the potential functional redundancy of this 17-member gene family and the early lethality of some members in the family (Hoshikawa et al. 1998; McWhirter et al. 1997; Miyake et al. 1998; Smallwood et al. 1996; Verdier et al. 1997; Yamasaki et al. 1996).

FGF receptor gene family

Functions of FGFs are mediated by several different types of receptors, including high-affinity receptors, which are a group of four transmembrane proteins with intrinsic tyrosine kinase activity (Basilico and Moscatelli 1992; Goldfarb 1996; Johnson and Williams 1993; Szebenyi and Fallon 1999). FGF receptors share several common structural features, including a hydrophobic leader sequence, three immunoglobulin (Ig)-like domains, an acidic box, a CAM (cell adhesion molecule) homology domain, a transmembrane region, and a divided tyrosine kinase domain (Green et al. 1996; Hou et al. 1991; Johnson et al. 1990, 1991; Mason 1994; Werner et al. 1992; Williams et al. 1994; Xu et al. 1992; and Fig. 1). These receptors normally exist as inactive monomer forms and become dimerized upon the binding to FGFs. The dimerization activates their intrinsic tyrosine kinase activity and triggers downstream effects through as yet unclarified signal transduction pathways. An interesting feature of this gene family is the existence of numerous mRNA isoforms generated by alternative splicing in the extracellular, juxtamembrane, and intracellular do-

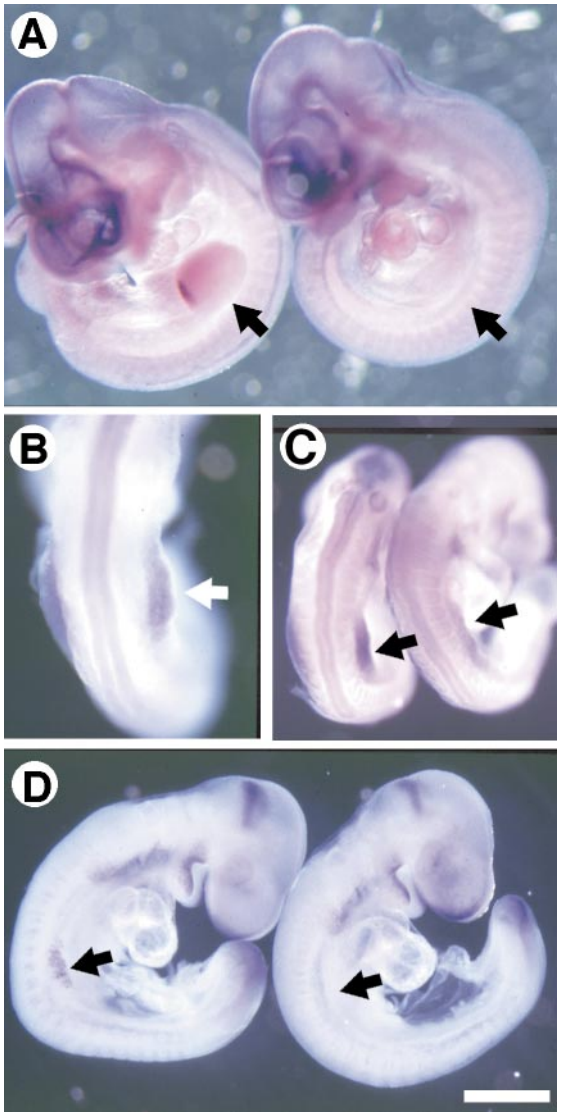

Fig. 2A–D Targeted deletion of FGFR2 results in failure of limb bud initiation. **A** Absence of limb bud in a E10.5 mutant embryo (*right arrow*). Both embryos were stained with a riboprobe for *Shh*. The positive-stained area in the control embryo (*left arrow*) is the ZPA. **B** *Fgf10* transcripts are detected in the mesenchyme of initiating limb bud. Its expression is significantly downregulated in the presumptive limb field of $Fgfr2^{\Delta IgIII/\Delta IgIII}$ embryos (*arrow* in **B**). **C** *Fgf10* is also expressed in the hindlimb bud at the earliest stages of initiation (E7.75). Downregulation of *Fgf10* is also observed in mutant hindlimb buds (not shown). **D** *Fgf8* expression in limb bud of early E9.5 control embryos (*left arrow*). Its expression is not detected in the limb field of all mutant embryos (*right arrow*). *Bars* 1000 μm (**A**); 310 μm (**B**); 520 μm (**C, D**)

mains (reviewed in Givol and Yayon 1992). For example, alternative splicing at exon 3, which encodes the entire Ig domain I (IgI), generates a number of isoforms, including full-length isoforms that contain three Ig loops [termed α (Hou et al. 1991) or A isoforms (Johnson et al. 1991)], and shorter isoforms that contain two Ig loops [termed β (Hou et al. 1991) or B isoforms (Johnson et al. 1991)]. Splicing in exon 8 and 9 yields the IIIb- and IIIc-containing isoforms, which have been shown to have differential ligand-binding specificity (Chellaiah et al. 1994; Werner et al. 1992). To identify the FGF receptors that are involved in limb initiation, we have examined expression of all four known receptors and found that *Fgfr1* and *Fgfr2* are the only receptors that showed significant expression at stages prior to AER formation. *Fgfr1* expression is exclusively in the limb mesenchyme [(Orr-Urtreger et al. 1991; Peters et al. 1992) and our own observation)], whereas *Fgfr2* is concentrated in the limb surface ectoderm, and is present at low levels in the mesenchyme adjacent to the ectoderm (Orr-Urtreger et al. 1991, 1993; Peters et al. 1992; Xu et al. 1998). Functions of all four FGF receptors were studied in mouse using targeted gene disruption. It was shown that embryos lacking FGFR3, FGFR4, or both have normal limbs (Colvin et al. 1996; Deng et al. 1996; Weinstein et al. 1998). These observations are consistent with the lack of expression of these genes in the early developing limbs. In contrast, embryos deficient for FGFR1 or FGFR2 died at embryonic day 7.5–9.5 (E7.5–9.5) or E4.5–5.5, respectively (Arman et al. 1998; Deng et al. 1994; Yamaguchi et al. 1994) (Table 1). Thus, it is impossible to use these null embryos to study FGF receptor functions during limb development. To overcome these difficulties, we and others have been using hypomorphic mutations, isoform knockouts, and transgenic and chimeric animal formation to manipulate mouse embryos. These studies allow us to assess functions of FGF receptors during limb development.

FGFR2 is essential for limb bud initiation

FGFR2 has been shown to be essential for limb bud initiation by several recent studies. The first was the expression of a dominant negative (DN) FGFR2b gene (Celli et al. 1998). This experiment differed from other studies using dominant-negative (DN) FGFR genes in that it used a soluble derivative of the FGFR2b gene. The rationale was that the soluble dominant negative gene product would bind FGFs in the extracellular matrix and prevent them from interacting with the endogenous FGF receptors, which it was shown to do in vitro. Indeed, in a comparison of soluble versus membrane-bound dominant negative FGFR genes, the soluble one was considerably more efficacious in generating observable phenotypes.

Expression of this soluble DN-FGFR2b was driven by the metallothionine promoter (MTp), which is active in a broad variety of tissues during midgestation development. Agenesis or dysgenesis was seen in multiple organs and tissues, including the limb buds. The onset of transcription from the MTp varied from embryo to embryo, and the phenotypes observed varied as a result. However, some limbless embryos were seen, as well as embryos with distal truncations in limb elements. Interestingly, hindlimb truncations were always more proximal than those seen in the forelimb, presumably because the hindlimb forms later in development when the MTp is more likely to be actively producing soluble DNFGFR. Indeed some transgenic hindlimbs formed only a rudimentary pelvis.

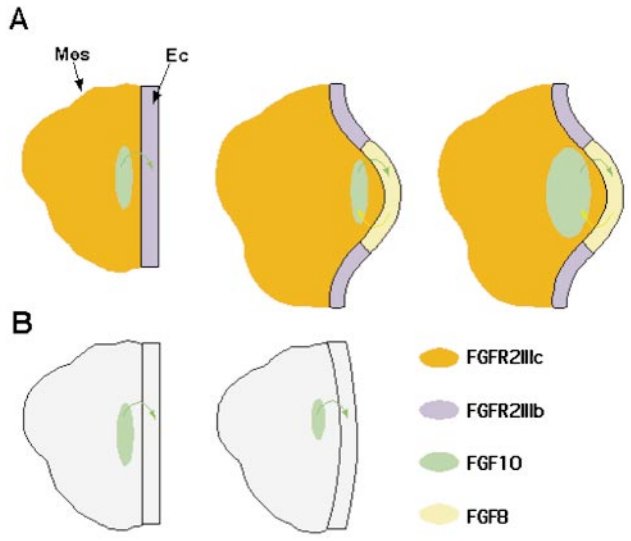

Fig. 3 A reciprocal signaling model showing the essential role of FGFR2 in epithelial-mesenchymal interactions during limb bud initiation. **A** Prior to limb bud initiation (before E9.25) in normal mouse embryos, *Fgf10* transcripts are detected in the mesenchyme (*Mes*) of the presumptive limb field. *FGFR2b* and *FGFR2c* are differentially expressed in the surface ectoderm (*Ec*) and the underlying mesenchyme, respectively. *FGF10* induces *Fgf8* expression in the overlying surface ectoderm through the activation of the ectodermally expressed FGFR2b, and initiates outgrowth of the limb bud. Once it is induced, FGF8 in the ectoderm interacts with the mesodermally expressed FGFR2c to maintain *Fgf10* expression and promote continuous proliferation of the underlying mesenchyme. Apical ectodermal ridge (AER) is subsequently induced by signals from proliferating mesenchyme. **B** In $Fgfr2^{\Delta IgIII/\Delta IgIII}$ embryos, expression of *Fgf10* in the mesenchyme cannot induce *Fgf8* in the overlying ectoderm, suggesting an essential role of FGFR2 in this process. Without FGF8 signals from ectoderm, *Fgf10* expression is gradually diminished in the mutant mesoderm of the presumptive limb field. Uncoupling of this epithelial-mesenchymal signaling loop due to the loss of FGFR2 halts the proliferation of mesenchymal cells at the earliest stages of limb induction and generates limbless embryos

A second report tying FGFR2 to limb bud induction was a presumably hypomorphic disruption of FGFR2. Xu et al. (1998) deleted exons 7, 8, and 9, which encode the third Ig loop of FGFR2. This is in contrast to a knockout of FGFR2 described by Arman et al. (1998), in which exons 9–12 were removed. In the latter study, a part of IgIIIc, the transmembrane domain, and part of the tyrosine kinase domain were deleted, resulting in peri-implantation lethality. The areas deleted in these two studies are shown in Fig. 1B.

The deletion created by Xu et al. (1998) eliminated IgIII ($Fgfr2^{\Delta IgIII}$), which is thought to determine the specificity of FGF binding (Chellaiah et al. 1994; Werner et al. 1992). Indeed, the mutant protein failed to bind to FGF in vitro, suggesting it had lost its FGF-binding activity. However, mice homozygous for this mutation survived until E10.5 and formed quasi-normal embryos, which died because of a placentation defect. These results imply that either the $Fgfr2^{\Delta IgIII}$ mutant had some residual FGF-binding activity, or that FGFR2 has FGF-independent functions. The latter is a distinct possibility, as both FGFR1 and FGFR2 possess other functional domains as discussed earlier. Although the FGFR2 mutant embryos generated by Xu et al. (1998) were relatively normal in both size and appearance, they lacked limb buds at all the stages of development examined (Fig. 2A).

FGFR2 mediates a reciprocal regulation loop between FGF8 and FGF10

Ohuchi et al. (1997) suggested the existence of a regulatory loop between FGF8 and FGF10, both of which are expressed during limb development. Cells that secrete FGF10 are capable of inducing ectopic limbs when implanted in the flank of chicken embryos, and can also induce expression of both FGF8 and FGF10. Cells that express FGF8 carry out the reciprocal regulation, that is, they induce the expression of FGF10. During normal chick development, FGF10 expression was shown to occur in the mesenchyme before the onset of both FGF8 expression and limb bud outgrowth.

Xu et al. (1998) expanded these results by first showing that the expression patterns of FGF8 and FGF10 in the mouse limb bud are the same as that seen in chicken. They show that FGF10 is expressed in the $Fgfr2^{\Delta IgIII/\Delta IgIII}$ mutants, although at a lower level than in normal embryos (Fig. 2B,C). However, FGF8 is not found in the limb field of the mutant embryos (Fig. 2D).

These results suggested that the reciprocal regulation loop between FGF10 and FGF8 was mediated by FGFR2, and was disrupted in the $Fgfr2^{\Delta IgIII/\Delta IgIII}$ mutants. This regulation was shown to be dependent on the differential expression of the FGFR2 isoforms, FGFR2b and FGFR2c. FGFR2b is expressed in the AER, but binds the mesenchymally expressed FGF10 to the exclusion of FGF8. FGFR2c is localized to the mesenchyme, but binds the FGF8 ligand produced in the AER. These results suggested a model, shown in Fig. 2, in which FGF10 is made in the mesenchyme of the limb field. It then diffuses into the ectoderm, where it binds FGFR2b and induces FGF8 in the ectoderm. The FGF8, in turn, diffuses into the mesoderm and activates FGFR2c, which causes the upregulation of FGF10. The FGF10 then continues the loop, while downstream activities result in limb bud induction.

Consistent with this model, a recent study showed that mouse embryos lacking FGF10 were limbless and did not express FGF8 in the ectoderm of the presumptive limb field (Min et al. 1998).

Loss of FGFR1 results in a distal truncation of mouse limb bud

Embryos null for FGFR1 died during gastrulation, displaying growth defects and axial mesoderm disorganization (Table 1). This early lethality leaves uncertainty about the role of this gene in limb development. To study functions of

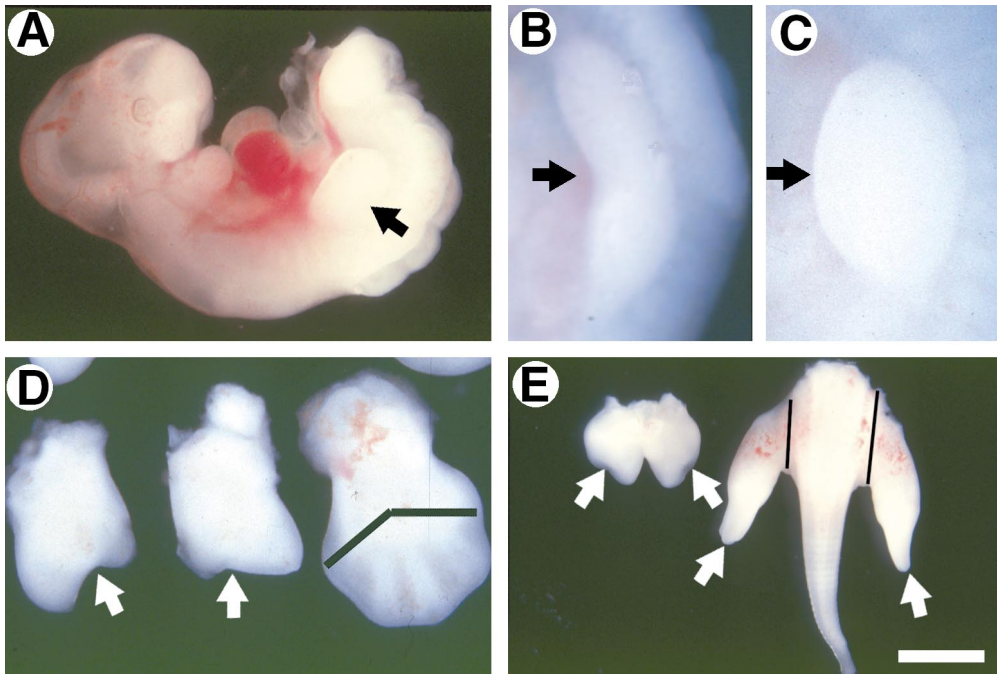

Fig. 4A–E Distal truncation of limb buds in *Fgfr1*$^{ex3/ex3}$ embryos. **A** An E11.5 *Fgfr1*$^{ex3/ex3}$ embryo. The mutant embryos could initiate limb buds (*arrow*), and survived significantly longer than FGFR1-null embryos reported previously (Deng et al. 1994; Yamaguchi et al. 1994). **B,C** E10.5 mutant (**B**) and control (**C**) limb buds. The mutant bud was wider along the A-P axis and had an indentation in the P-D axis (*arrows*). **D** E12.5 mutant and control limb buds. The mutants exhibited a distal truncation and an indentation (arrows). *The line on the control limb bud* marks the truncation level in the mutant buds. **E** E12.5 mutant (*left*) and control hindlimb buds (*arrows*). The mutant buds stacked together due to a lack of an axial element and a tail. *The lines on the control limb bud* mark the axial elements that are missed in the mutant buds. *Bars* 1000 μm (**A**); 400 μm (**B,C**); 660 μm (**D**); 1.6 mm (**E**)

FGFR1 further, chimeric mice formed between wild-type embryos and FGFR1-null embryonic stem (FGFR1$^{-/-}$) cells were analyzed (Ciruna 1997; Deng et al. 1997). These studies revealed an essential function of FGFR1 in morphogenetic cell movements, and our study indicated that FGFR1 is also involved in limb outgrowth. The distribution of FGFR1$^{-/-}$ cells was found to be biased to the surface ectoderm and AER, where this gene is not normally expressed (Deng et al. 1997). FGFR1-deficient cells could also be found in the limb bud mesenchyme, but were excluded from the progress zone. At E11.5–E12.5, all chimeric limb buds were developmentally retarded, and exhibited an abnormal shape and rough surface. These abnormalities might be caused by unbalanced growth of host and donor cells, since the donor cells are segregated away from fast-growing areas such as the progress zone and the tips of the digits (Deng et al. 1997). These observations suggested an indispensable function of FGF/FGFR-1 signals in the growth and patterning of the developing limb bud.

However, because of the nature of the random distribution of ES cells, it is extremely difficult to obtain a sufficient number of chimeric limb buds for further study. To extend our understanding of functions in limb development, we disrupted the full-length isoform of FGFR1 (α isoform) by insertion of a neomycin gene into exon 3 of the *Fgfr1* gene (Fig. 1). Embryos homozygous for the mutation (*Fgfr1*$^{ex3/ex3}$) did not express the α isoforms as revealed by both Northern blot and Western blot analysis, and died at embryonic days 9.5–12.5 due to multiple abnormalities associated with posterior embryonic development (Fig. 4A, and Xu et al. 1999). The extended survival of mutant embryos makes it possible to study FGFR1 function in limb development. We found that all mutants of E9.5 and older developed forelimb buds. However, the mutant buds were often shorter in the P-D axis and wider along the A-P axis compared with limb buds from normal embryos (Fig. 4B,C). In most cases, an indentation at the tip of the bud was observed (Fig. 4B). At E12.5, all mutant limb buds studied (n>20) were fork shaped, presumably as a consequence of the indentation and the distal truncation (Fig. 4D). Hindlimb buds, which normally become visible at around E9.75, were absent in over 80% of the mutant embryos, which exhibited severe posterior disorganization. However, they were present in the remaining *Fgfr1*$^{ex3/ex3}$ embryos, which were less severely affected by the mutation, including all E12.5 mutant embryos (n=9). The mutant hindlimb buds exhibited the same morphological defects as forelimb buds (Fig. 4E). Interestingly, the mutant hindlimb buds often grew together due to the absence of axial elements. These observations indicated that loss of FGFR1α isoforms does not block limb bud initiation; however, it does affect limb patterning.

FGFR1 and progress zone activity of mouse limb buds

To study limb development further, we analyzed expression patterns of a number of molecular markers that are expressed along P-D and/or A-P axes. Mutant limb buds contained the AER as indicated by probes for *Fgf8* (Fig. 5A) and *Fgf4* (not shown). However, the level of *Shh* expression in the zone of polarizing activity (ZPA) in the mutant limb buds was significantly lower than controls (Fig. 5B). It was

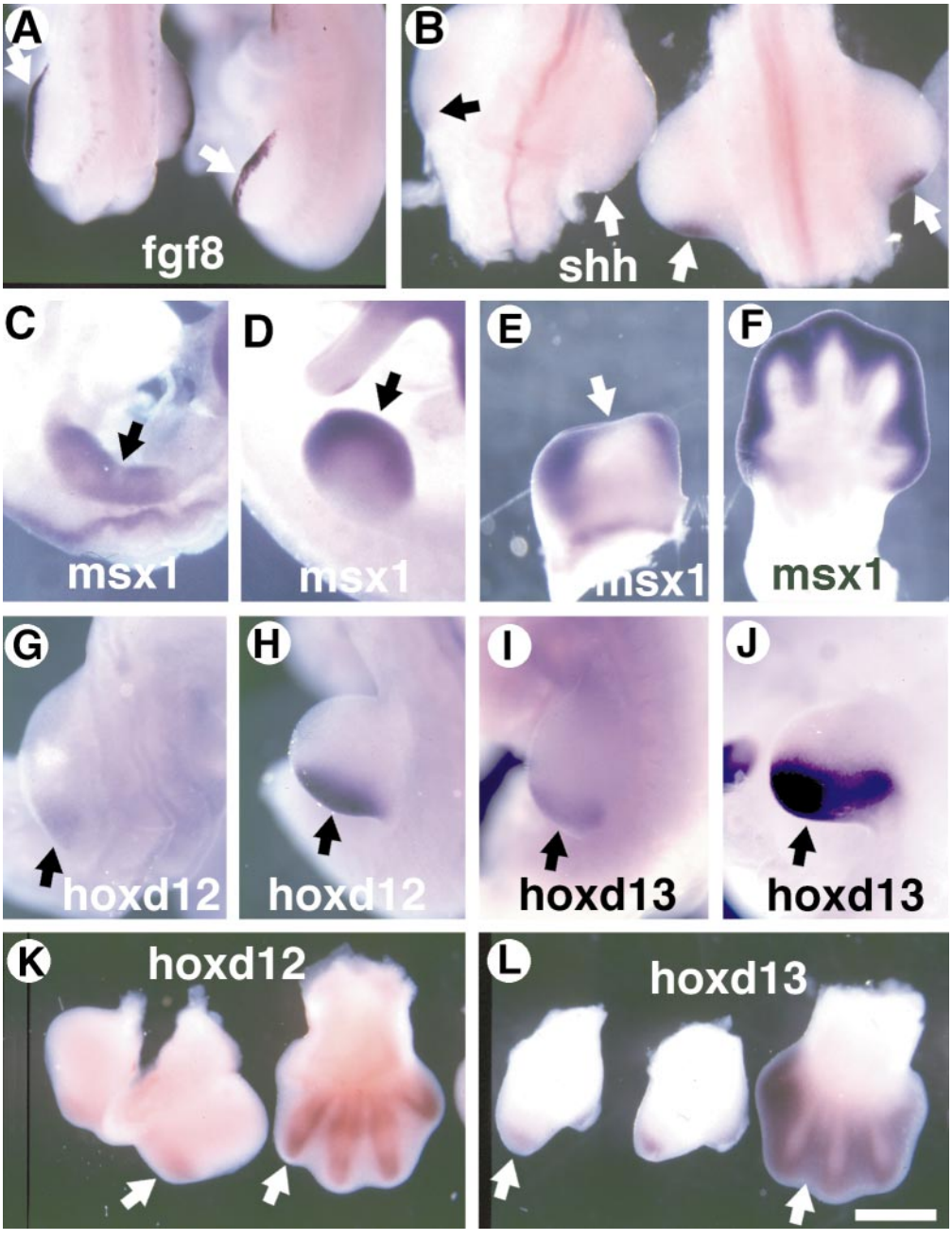

Fig. 5 Molecular analysis of limb defects in *Fgfr1*$^{ex3/ex3}$ embryos. **A** *Fgf8* expression in E9.5 limb buds (*arrows*). Mutant buds are *on the left* and controls are *on the right*. **B** *Shh* expression in E10.5 mutant and control ZPA (*arrows*). **C–F** *Msx1* expression in E10.5 (**C,D**), E12.5 (**E,F**) mutant (**C,E**) and control limb buds (**D,F**). Notice the distal expression of *Msx1* is weaker at E10.5 (**C**, *arrow*) and disappeared at E12.5 (**E**, *arrow*) mutant embryos. **G–J** *HoxD12* (**G,H**) and *HoxD13* (**I,J**) expression in E10.75 mutant (**G,I**) and control (**H,J**) limb buds. Both *HoxD12* and *HoxD13* were significantly weaker in mutant buds. **K,L** *HoxD12* (**K**) and *HoxD13* (**L**) expression in E12.5 buds. *HoxD12* and *HoxD13* are expressed in the digits and mesenchyme between digits in control buds, respectively. They are virtually absent in the mutants. *Bars* 400 μm (**A,B**); 660 μm (**C,D, G–L**); 550 μm (**E,F**)

shown that FGF signals regulate *Msx1* expression in the progress zone of the limb bud (Wang and Sassoon 1995). We found that the *Msx1* expression along the tip of the E10.5 mutant limb buds was weaker than in the control buds (Fig. 5C,D), although no apparent difference was detected between E9.5 mutant and control limb buds (not shown). 5' *HoxD* cluster genes are thought to play an important role in distal limb development and patterning (Morgan et al. 1992; Tabin 1992). Down-regulation of both *HoxD12* (Fig. 5G,H) and *HoxD13* (Fig. 5I, J) was found in mutant limb buds, whereas the expression of *HoxD11* was not affected (not shown). In the E12.5 embryos, *HoxD12* (Fig. 5K) and *HoxD13* (Fig. 5L) are differentially expressed in newly formed digits and surrounding mesenchyme. The *Fgfr1*$^{ex3/ex3}$ embryos do not contain any digits as revealed by the staining of both these markers (Fig. 5K,L), suggesting that the loss of FGFR1α isoforms completely blocked distal development of the initiating limb buds.

Distal truncations were observed in chick limb buds whose AER were surgically removed (Fallon and Hall 1994; Niswander and Martin 1993; Niswander et al. 1993; Saunders 1948). Because the exogenous application of FGFs to limb buds from which the AER has been excised can restore their normal growth and patterning (Fallon and Hall 1994; Niswander and Martin 1993; Niswander et al.

Table 1 Mutational analysis of FGFs and FGFRs in mouse

Genes	Mutations	Phenotype	References
FGF2	Disruption/Null	Neuronal defects; Normal development of limb	Ortega et al. 1998; Dono et al. 1998; Zhou et al. 1998
FGF3	Disruption/Null	Inner ear defect, postnatal lethal; Normal development of limb	Mansour et al. 1993
FGF4	Disruption/Null	Epiblast cells defects, lethal at E5.5	Feldman et al. 1995
FGF5	Disruption/ Null	Hair follicle defects, survive to adulthood; Normal development of limb	Hebert et al. 1994
FGF6	Disruption/Null	Muscle regeneration; Normal development of limb	Floss et al. 1997
FGF7	Disruption/Null	Poor keratinocyte organization; Normal development of limb	Guo et al. 1996
FGF8	Disruption/Null	Primitive streak defects, lethal at E8.5	Meyers et al. 1998
FGF9	Disruption/Null	Normal development of limb	D. Ornitz, personal communication
FGF10	Disruption/Null	Absence of limbs and lung	Min et al. 1998
FGFR1	Disruption/Null	Growth retardation, defect of mesodermal patterning, lethal at E7.5–9.5	Deng et al. 1994 Yamaguchi et al. 1994
FGFR1	FGFR1-deficient ES	Defective cell migration through primitive streak; malformation of chimeric limb buds	Ciruna et al. 1997; Deng et al. 1997
FGFR1	Disruption/α isoforms	Distal trunction of limb bud, lethal at E9.5.–12.5 due to posterior embryonic defects	Xu et al. 1999, and this study
FGFR2	Deletion/Null	Lethal at E4.5–5.5	Arman et al. 1998
FGFR2	Deletion of IgIII/ Hypomorphic	Failure of limb bud initiation and placenta formation, lethal at E10.5	Xu et al. 1998
FGFR2	Transgene dominant Negative	Agenesis or severe dysgenesis of multiple organs, including limb	Celli et al. 1998
FGFR3	Disruption/Null	Bone over growth, inner ear defect	Colvin et al. 1996; Deng et al. 1996
FGFR4	Disruption/Null	Normal	Weinstein et al. 1998
FGFR3/ FGFR4	Cross of FGFR3 and FGFR4 mutants	Neonatal growth retardation, lung abnormalities	Weinstein et al. 1998

1993), we reasoned that the distal truncation is a direct consequence of the block of FGF signals in the FGFR1α-deficient limbs. Thus, our study revealed an important function of the FGFR1α isoforms in mediating FGF signals from the AER to specify P-D axis formation. Based on the following two observations, we believe that FGFR1-mediated signals are required for maintaining ZPA and progress zone activities. First, it is known that *Msx1* is expressed in the progress zone and is thought to keep cells in a proliferating and undifferentiated state (Song et al. 1992; Vogel et al. 1995a). *Msx1* expression is downregulated in E10.5 mutant limb buds, suggesting that the mutant progress zone has a lower proliferation profile. By E12.5, the expression domain of *Msx1* in distal limb buds completely disappeared (Fig. 5E,F), indicating a lack of the progress zone in mutant limb buds. In addition to the diminished progress zone activity, the FGFR1α mutant limb buds also exhibited defects in the A-P axis, as reflected by the downregulation of *Shh*, which is known to be essential in the establishment of the A-P axis (Chiang et al. 1996; Pagan et al. 1996; Riddle et al. 1993). It has recently been shown that SHH is also involved in P-D identity (reviewed in Johnson and Tabin 1997). In-

terestingly, the distal truncation of FGFR1α-deficient limb buds resembles that found in SHH-deficient mice (Chiang et al. 1996), suggesting a functional interaction between FGF signals and SHH in limb growth along the P-D axis. Since it is known that both FGF and SHH can affect the expression of the 5' *HoxD* genes, the downregulation of *HoxD12* and *HoxD13* may be directly linked with the loss of FGF/FGFR1α signals or may be due to the downregulation of *Shh*.

More recently, a much milder effect of FGFR1 mutation on limb development was reported based on analysis of mutant embryos created by a *neo* gene insertion into introns of the *Fgfr1* gene (Partanen et al. 1998). In this case, the reduced FGFR1 function is responsible for a variety of limb abnormalities. The most severely affected embryos exhibited distal truncation whereas the less affected embryos survived up to birth and exhibited syn- and oligodactyly, delayed ossification of distal phalanges, and postaxial cartilage condensation. Altogether, these studies revealed an essential role of FGF/FGFR1 signals in all stages of the limb development rather than its initiation, which is mainly controlled by the FGFR2-mediated signals (Xu et al. 1998).

Limb anomalies associated with missense mutations in human FGFRs

Evidence that FGF receptors may be involved in the later stages of limb development comes from the correlation of FGFR mutations with a number of human skeletal dysplasias, including Apert, Pfieffer, Jackson-Weiss, Crouzon, and Barre-Stevenson Cutis Gyrata syndromes (Fig. 1). All of these result in craniosynostosis, as well as malformations of the hands and/or feet, including broad thumbs, big toes, and soft tissue syndactyly (reviewed by Muenke and Schell 1995). It is unlikely that these diseases are a result of haploinsufficiency, as none of the mouse mutants described earlier exhibits abnormalities of the digits in heterozygotes (Deng et al. 1994; Xu et al. 1998; Yamaguchi et al. 1994). Indeed, these are thought to be the result of increased FGF/FGFR signals, as several studies showed ligand-independent activation of these mutant FGF receptors in vitro (Galvin et al. 1996; Neilson and Friesel 1995, 1996; Park et al. 1995; Robertson et al. 1998). Consistently, it was recently reported that upregulation of *Fgf3* and *Fgf4* by a retroviral insertion resulted in a phenotype in mice that resembled Crouzon syndrome in humans (Carlton et al. 1998).

However, it is not clear how the increased receptor activity could cause hand and foot abnormalities. In mouse, it has been shown that both *Fgfr1* and *Fgfr2* are expressed in the distal tips of the early developing limb buds. These receptors must provide crucial mitotic stimuli for limb mesenchymal proliferation, since the targeted deletion of FGFR2 (Xu et al. 1998) and FGFR1α isoforms (this study) resulted in failure of limb initiation and distal truncation, respectively. The rapid cell proliferation during early stages of digit formation is accompanied by massive apoptosis in the cells between digits. This process is followed by continued cell proliferation and death, and skeletal differentiation. Apparently, the separation of adjacent skeletal elements in the hand and foot requires a balance between proliferation, differentiation and programmed cell death. It is conceivable that missense mutations in FGF receptors may affect either one or all of these activities, leading to the observed malformations. The elucidation of mechanisms underlying these diseases may require the analysis of mouse models carrying the mutations found in human patients.

Conclusions and future directions

Fibroblast growth factors have been considered primary inductive signals in vertebrate limb induction because of their ability to replace the AER and to induce formation of a complete limb in chick (Cohn et al. 1995; Crossley et al. 1996; Fallon et al. 1994; Niswander and Martin 1993; Niswander et al. 1993; Ohuchi et al. 1997; Vogel et al. 1996). The present mutational analysis of FGF receptors provides direct genetic evidence that FGF signals are absolutely required for development of vertebrate limbs. The loss of FGFR2 uncouples the FGF8-FGF10 reciprocal activation loop, generates limbless embryos, and provides molecular clues concerning the functions of FGF signals underlying the earliest stages of limb initiation. The loss of FGFR1α isoforms, which results in distal truncation, reveals an essential role of FGF/FGFR1α signals in limb outgrowth along the P-D axis. The downregulation of signals in the progress zone and ZPA reinforced the notion that coordinated efforts of signals from all three axes are required for the correct development and patterning of the initiating limbs. Indeed, recent studies have revealed the involvement of multiple factors that are not discussed in this review, such as T-box genes (Gibson-Brown et al. 1998), Hox genes (Logan et al. 1998; Nelson et al. 1996), retinoic acid (Lu et al. 1997), Wnt genes (Kengaku et al. 1998) and bone morphogenetic proteins (BMPs) (Zou et al. 1997), in limb development and patterning. The functional interaction of FGF/FGFR signals with these molecules during all phases of limb development, including initiation, outgrowth, digit and bone formation, and patterning of soft tissues, will be one of the future directions of this fast and most exciting field.

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