Conditional inactivation of Fgfr1 in mouse defines its role in limb bud establishment, outgrowth and digit patterning

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Summary

Previous studies have implicated fibroblast growth factor receptor 1 (FGFR1) in limb development. However, the precise nature and complexity of its role have not been defined. Here, we dissect Fgfr1 function in mouse limb by conditional inactivation of Fgfr1 using two different Cre recombinase-expressing lines. Use of the T (brachyury)-cre line led to Fgfr1 inactivation in all limb bud mesenchyme (LBM) cells during limb initiation. This mutant reveals FGFR1 function in two phases of limb development. In a nascent limb bud, FGFR1 promotes the length of the proximodistal (PD) axis while restricting the dimensions of the other two axes. It also serves an unexpected role in limiting LBM cell number in this early phase. Later on during limb outgrowth, FGFR1 is essential for the expansion of skeletal precursor population by maintaining cell survival. Use of mice carrying the sonic hedgehog (Shh) allele led to Fgfr1 inactivation in posterior LBM cells. This mutant allows us to test the role of Fgfr1 in gene expression regulation without disturbing limb bud growth. Our data show that during autopod patterning, FGFR1 influences digit number and identity, probably through cell-autonomous regulation of Shh expression. Our study of these two Fgfr1 conditional mutants has elucidated the multiple roles of FGFR1 in limb bud establishment, growth and patterning.

Key words: Cre-mediated recombination, FGF, Fgfr1 signaling, Limb development, Mouse, Patterning, Shh

Introduction

Mouse limbs initiate as limb buds that protrude from set positions along the side of the embryo. Early limb buds consist of an ectoderm-derived epithelial jacket encasing morphologically undifferentiated mesoderm-derived mesenchymal cells. Signaling between these two cell lineages is important for limb development along its three axes: the proximodistal (PD or shoulder to fingertip) axis, the anteroposterior (AP or thumb to little finger) axis and the dorsoventral (DV or back of hand to palm) axis (Niswander, 2003). In particular, the apical ectodermal ridge (AER), a specialized group of ectodermal cells that rims the growing tip of the limb bud, emits signals for the growth and patterning of the underlying mesenchyme. Key signals involved in this process are fibroblast growth factors (FGFs) and several Fgf genes are expressed in the AER (AER-Fgfs). Limb-specific inactivation of two AER-Fgfs, Fgf4 and Fgf8, leads to severe reduction of skeletal elements in all three limb segments along the PD axis (Boulet et al., 2004; Sun et al., 2002). Analysis of these mutants led to the proposal that AER-FGFS are important for the expansion of the skeletal precursor population for each segment of the limb (Sun et al., 2002). The exact mechanism by which AER-FGFS direct cellular changes in the underlying mesenchyme is unclear. There are four mouse FGF receptors (FGFRs), each characterized by three extracellular immunoglobulin (Ig)-like domains, a single transmembrane domain and an intracellular split cytoplasmic tyrosine kinase domain (Itoh and Ornitz, 2004). Alternative splicing within the third Ig loop of FGFR1-3 produces two splice variants: IIIb and IIIc which display different ligand specificities. For example, mitogenic assays in cultured cells show that FGF4 and FGF8 preferentially activate the IIIc isoform of FGFR (Ornitz et al., 1996). Conversely, genetic data suggest that FGF10, which is expressed in the LBM, preferentially activates the IIIb isoform (Min et al., 1998; Revest et al., 2001; Sekine et al., 1999). In addition, the IIIb and IIIc isoforms are often differentially expressed, with IIIb preferentially in the epithelium and IIIc in the mesenchyme (Finch et al., 1995; Orr-Urtreger et al., 1993; Peters et al., 1992).

Two Fgfrs, Fgfr1 and Fgfr2 are expressed in the early limb bud (Orr-Urtreger et al., 1993; Peters et al., 1992; Xu et al., 1998; Yamaguchi et al., 1992). Fgfr2-IIIc, although expressed in early LBM, is only essential at a later stage in ossification (Eswararama et al., 2002; Yu et al., 2003). A role for FGFR1 in limb has been implicated through studies of chimera and hypomorphic mutants, which bypass the gastrulation defect that causes Fgfr1-/- mutants to die prior to limb initiation (Ciruna and Rossant, 2001; Deng et al., 1994; Yamaguchi et
These milder mutants exhibit deformed limb buds and varying degrees of reduction in limb skeletal elements (Deng et al., 1997; Partanen et al., 1998; Xu et al., 1999). However, the precise role of FGFR1 in limb formation awaits definition. Here, we dissect FGFR1 function by conditional inactivation in mouse using the Cre/loxP approach. Our results show that FGFR1 plays multiple roles in limb bud establishment, outgrowth and patterning.

Materials and methods

Generation of Fgfr1 limb mutants

Mice carrying a conditional allele of Fgfr1 (Fgfr1<sup>cre</sup>/Fgfr1<sup>co</sup>) (Xu et al., 2002) were mated to either Tcre line (Perantoni et al., 2005) or Shhcre<sup>+/−</sup> (Harfe et al., 2004) allele to generate Tcre;Fgfr1<sup>cre</sup> and Shhcre;Fgfr1<sup>cre</sup> mutant embryos, respectively. Offspring were genotyped using the following PCR primer pairs: for Cre, 5’-TGATGAGGTTGCCGCAAGACC-3’ and 5’-CCATGAGTGAAACCTGGAAG-3’; for Fgfr1, 5’-CTG-GTATCCGTGACTATC-3’ and 5’-CAATCTGATCCAGACAC-3’.

RT-PCR analysis

For normal and Tcre;Fgfr1 mutant limb buds (two pairs each), the LBM was dissected from the ectoderm and total RNA was prepared using TRIzol (Invitrogen). First-strand synthesis was carried out using the Superscript First Strand cDNA Synthesis Kit (Invitrogen). PCR was performed using the following primer pairs: for Fgfr1, 5’-TCTGAAGCCCTGGAAGAGAGA-3’ and 5’-TTCTAGAGGCCAGAATGACTCAT-3’; for Gapdh, 5’-ACCACATCGTCCATGCAC-3’ and 5’-TCCACACCCCTGTGTGTA-3’.

Embryo isolation and phenotype analyses

Embryos were dissected from time-mated mice, counting noon on the day the vaginal plug was found as embryonic day (E) 0.5. Whole-mount in situ hybridization was performed as previously described (Neubauer et al., 1997). The Fgfr1 in situ probe was prepared from a plasmid containing Fgfr1 exon 9 cDNA. This cDNA was generated by PCR using primer pair: 5’-TCTGGAAGGCTGGAGAAGAGAGA-3’ and 5’-TTCTAGAGGCCAGAATGACTCAT-3’. LIMb buds for histological analysis were fixed in 4% paraformaldehyde after whole-mount in situ hybridization and stained with LysoTracker Red (Molecular Probes) using a modified protocol (Zucker et al., 2004).

Areas of cell death were detected by staining with LysoTracker Red DND-99 (Molecular Probes) using a modified protocol (Zucker et al., 2004) and in accordance with published expression data (Orr-Urtreger et al., 1993; Xu et al., 1998). Using the same analysis, in Shhcre;Fgfr1<sup>cre</sup>, we can detect a domain lacking Fgfr1 expression in the posterior mesenchyme of E10.5 limb buds, shortly after the commencement of Shh<sup>cre</sup> activity (Fig. 1D,G,H).

Results

Two Cre recombinase lines are used to inactivate Fgfr1 in the LBM

By whole-mount RNA in situ hybridization, we found that Fgfr1 is widely expressed in the lateral plate mesoderm (LPM), including the LBM prior to as well as after limb initiation (Fig. 1E; data not shown), in agreement with previous findings (Orr-Urtreger et al., 1991; Peters et al., 1992; Yamaguchi et al., 1992). To by-pass the gastrulation-stage lethality of Fgfr1 null allele (Ciruna and Rossant, 2001; Deng et al., 1994; Yamaguchi et al., 1994), we inactivated Fgfr1 using an existing Fgfr1 conditional allele, Fgfr1<sup>cre</sup> (Xu et al., 2002). Upon Cre-mediated recombination of this allele, Fgfr1 exons 8-14 are deleted, resulting in a null allele in which both the IIIb and IIIc isoforms are inactivated. We generated two Fgfr1 mutants using two Cre-expressing lines, the Tcre transgenic line (Perantoni et al., 2005) and mice carrying the Shhcre<sup>Cre</sup> allele (Harfe et al., 2004). We found no difference in phenotype between cre;Fgfr1<sup>null</sup> or cre;Fgfr1<sup>Cre</sup>, and we therefore refer to these genotypes as Tcre;Fgfr1 or Shhcre;Fgfr1, depending on which Cre-expressing line was used. Tcre is generated through a transgenic approach using a 500 bp T (brachyury) promoter (Clements et al., 1996) driving Cre expression in the primitive streak-derived mesoderm lineages starting at E7.5 (Perantoni et al., 2005). By mating Tcre line to a cre activity reporter line (Soriano, 1999), we found that at E8.5 and E10.0, Tcre is active in the LPM posterior to the heart, including the LBM (Fig. 1A-C). As a result of Tcre function, Fgfr1 is completely inactivated in Tcre;Fgfr1 LBM at E10.0, supported by data from RT-PCR (Fig. 1M) and whole-mount in situ hybridization analysis using a probe hybridizing to the deleted region of Fgfr1 (Fig. 1E,F). To assess the impact on FGF signal reception, we assayed for the expression of genes regulated by FGFs, including sprouty (Spry) 2, Spry4 and Mkp3 (Dusp6 – Mouse Genome Informatics) (Eblaghi et al., 2003; Kawakami et al., 2003; Minowada et al., 1999). Their expression suggests that FGF signaling is markedly reduced, although not abolished, in the Tcre;Fgfr1 mutant hindlimb (Fig. 1I,J and Fig. 3S,T and data not shown). The most likely candidate to act redundantly with Fgfr1 to mediate this residual FGF signaling is Fgfr2. In Tcre;Fgfr1 mutant hindlimbs, we found that Fgfr2 is expressed normally compared with control (data not shown), and in accordance with previous published expression data (Orr-Urtreger et al., 1993; Xu et al., 1998).

Using the same analysis, in Shhcre;Fgfr1<sup>cre</sup>, we can detect a domain lacking Fgfr1 expression in the posterior mesenchyme of E10.5 limb buds, shortly after the commencement of Shh<sup>Cre</sup> activity (Fig. 1D,G,H). Spry4 and Mkp3 gene expression is reduced in this domain (Fig. 1K,L and Fig. 5G,H).

Inactivating Fgfr1 in LBM affects the size and shape of all three limb skeletal segments

Tcre;Fgfr1 mutants die at birth probably owing to neural tube and axial skeletal defects (see below). In E17.5 mutant forelimbs (n=8), we found that the stylopod is shortened by an average of 15%. The zeugopod, reduced by 12%, is often fused at the distal end (Fig. 2A,B, n=4/8 mutant forelimbs). The mutant autopod often consists of three digits, one triphalangeal digit flanked by two bi-phalangeal digits (Fig. 2E,F, n=5/8 forelimbs).

The Tcre;Fgfr1 mutant hindlimb is more severely affected than the forelimb (Fig. 2C,D,G,H). One explanation for the increased severity is based on a possible reduction of LPM cell number in the prospective hindlimb, but not forelimb region prior to limb initiation. This reduction is deduced from a combination of phenotypes, including irregular somite size and
an expanded open neural plate at E9.5, and misshapen ribs and axial vertebrae at E17.5 (data not shown). These defects are probably due to a previously described requirement of Fgfr1 in mesoderm production (Ciruna and Rossant, 2001; Ciruna et al., 1997; Deng et al., 1997; Yamaguchi et al., 1994). In Tcre;Fgfr1 hindlimb, a defect in mesoderm production will lead to reduced prospective LBM cell number, and in turn a reduced limb skeleton. By contrast, none of the phenotypes indicative of mesoderm reduction is observed within and rostral to the forelimb region, suggesting that the phenotypes observed in the forelimb are not compounded by earlier defects in mesoderm production. Thus, to address the specific role of FGFR1 in limb development, we have concentrated on the forelimb of the Tcre;Fgfr1 mutant for subsequent analyses.

Using Sox9 expression to outline the initial cartilage condensations, we found that a reduction in condensation size and number contributes to reduced forelimb skeletons in the Tcre;Fgfr1 mutant (Fig. 2I-L). In addition, we found that at E11.5, although five distinct condensations are observed in wild-type forelimb buds (Fig. 2I, n=4 limb buds), individual condensations cannot be discerned in the Tcre;Fgfr1 forelimb buds (Fig. 2I, n=4 limb buds). This suggests that separation of the digit condensations is delayed in Tcre;Fgfr1 mutant.

Based on phalanx number, the two bi-phalangeal digits in the Tcre;Fgfr1 forelimb might be of digit 1 character. To address whether this conclusion is also supported by molecular characteristics, we assayed for Hoxd12 and Hoxd13 expression (Fig. 2M-P). Previous studies show that digit 1 identity is marked by the absence of Hoxd12 and presence of Hoxd13 expression (Fromental-Ramain et al., 1996; Knezevic et al., 1997; Zakany et al., 1997). Their expression in Tcre;Fgfr1 mutant limb buds supports that the most anterior digit is digit 1, while the most posterior digit is not, despite being biphalangeal. These results show that in Tcre;Fgfr1 limbs, digit 1 is present while some of the posterior digits are absent.

Fgfr1 regulates nascent limb bud shape and cell number

We found that the Tcre;Fgfr1 forelimb bud is misshapen at E10.0 shortly after limb initiation. Although shorter along the PD axis, it is wider along the AP axis and thicker along the DV axis (Fig. 3A,B,E,F). By E10.5, the differences in all three axes are further exaggerated (Fig. 3C,D,G,H). A previous study of Fgfr1 hypomorphic mutants show that a posterior shift in Hoxb9 expression in the LPM may be responsible for the expansion of limb bud AP width (Partanen et al., 1998). A careful examination of Hoxb9 expression in Tcre;Fgfr1 LPM shows that there is no posterior shift of expression in this mutant to explain the increase in width (data not shown).

Using Fgf8 expression as a marker for AER, we found that in Tcre;Fgfr1 forelimb buds the AER is wider along DV axis starting at E10.0 (Fig. 3I-L) and shorter along AP axis starting...
Development of AER morphology in a cell non-autonomous manner. A possible mediator for this function is GREMLIN, a secreted antagonist of BMP signaling. *Gremlin* null mutants show a similar AER phenotype (Khokha et al., 2003; Michos et al., 2004), and its expression is downregulated in Tcre;Fgfr1 limb buds (Fig. 3M,N).

To address whether the whole limb bud shape change is accompanied by cell number changes, we counted LBM cell number (see Table S1 in the supplementary material). Our result shows that there is an approximate increase of 65% and 31% in the number of cells in the Tcre;Fgfr1 forelimb buds compared with controls at E10.0 and E10.5, respectively. At these stages, using phosphorylated Histone H3 antibody staining, no significant difference in cell proliferation is detected between mutant and normal to account for the increase in cell number (data not shown).

The initial excess cell number in Tcre;Fgfr1 limb buds is quickly negated by increased cell death observed starting at E10.5 (Fig. 3O,P). By E11.5, a mutant limb bud on average has 26% fewer cells compared with normal (see Table S1 in the supplementary material). We found that the expression of *Dkk1*, a mediator of limb bud cell death (Grotewold and Ruther, 2002; Mukhopadhyay et al., 2001), is increased in Tcre;Fgfr1 limb buds (Fig. 3Q,R). This provides a possible molecular mechanism for the cell death phenotype. Despite the increased number of dying cells, the death domain in Tcre;Fgfr1 limb buds is confined to the proximal and anterior LBM, similar to normal (Fig. 3O,P). We hypothesize that no cell death is detected in the distal mesenchyme because these cells are protected by residual FGF signaling, as indicated by *Spry4* expression (Fig. 3S,T).

**Complete inactivation of Fgfr1 in LBM affects the expression of key patterning genes**

In search of additional molecular changes, we found that the expression of *Shh* is reduced to a very small domain in Tcre;Fgfr1 forelimb buds (Fig. 4A,B). Accordingly, *Ptc1* and *Gli1* expression, which is responsive to the SHH signal, is detected in reduced domains (data not shown). In addition, *Alx4* expression, which is restricted by SHH signal to the anterior mesenchyme (Takahashi et al., 1998), is detected in a larger domain in the mutant (Fig. 4C,D). These results indicate that SHH signaling is reduced in the absence of FGFRI. The AER expression of all three BMP genes implicated in limb bud patterning, *Bmp2*, *Bmp4* and *Bmp7* is slightly upregulated in intensity in Tcre;Fgfr1 limb buds, possibly owing to the widened AER (Fig. 4E-H; data not shown). Interestingly, the mesenchymal expression of *Bmp4* and *Bmp7* is slightly reduced, while that of *Bmp2* is upregulated in the Tcre;Fgfr1 limb buds. This suggests that FGFRI regulates Bmp gene expression in a complex manner.

A previous study of a hypomorphic Fgfr1 mutant shows that *Hoxd13* expression is downregulated in those limbs (Partanen et al., 1998). Consistent with this, we found that in E10.5 Tcre;Fgfr1 limb buds, expression of both *Hoxd13* and the paralogous *Hoxa13* is reduced, with *Hoxa13* more severely downregulated (Fig. 4I-L). Interestingly, by E12.5 *Hoxd13* expression in Tcre;Fgfr1 limb buds appears to have recovered (compare Fig. 2P with Fig. 4L). A plausible explanation for this change is that early phase and late phase *Hoxd13* expression may be differentially controlled. This was also exemplified by a previous observation that in a limb-specific *Shh* chick mutant termed *oligozeugodactly* (*ozd*), early phase *Hoxd13* expression is absent while late phase expression is present (Ros et al., 2003). In summary, our molecular data suggest that a combination of these gene expression changes may account for the phenotypes in the Tcre;Fgfr1 mutant.

**Shhcre;Fgfr1 limb buds permit investigation of gene expression in the absence of growth defects**

The downregulation of *Shh* expression in Tcre;Fgfr1 limb buds supports the hypothesis of a transcriptional feedback loop between Fgf genes and *Shh* (Laufer et al., 1994; Niswander et
However, as the Tcre:Fgfr1 and all of the previously characterized AER-Fgf mutant limb buds are abnormal in size and shape (Boulet et al., 2004; Sun et al., 2002), it remains possible that any detected reduction or absence of gene expression is due to changes in cell number.

The Shhcre:Fgfr1 mutant offers several features essential for a rigorous test of FGFR1 function in gene expression regulation. First, this mutant presents a time window in which FGFR1 function in growth does not interfere with interpretation of data on gene expression. In Shhcre:Fgfr1 limb buds, total limb bud size as well as size of the Fgfr1 domain as outlined by Shh activity remains normal until E11.5 (Fig. 5A,B; data not shown). In addition, LysoTracker analysis indicates that there is no aberrant cell death at E10.75 and E11.5 (data not shown). These results suggest that in Fgfr1 limb buds younger than E11.5, Fgfr1 cells are normal in number and capable of appropriate gene expression. Second, the Shhcre:Fgfr1 mutant provides data on possible cell autonomous nature of gene expression regulation by FGFR1. In each Shhcre:Fgfr1 limb bud, Fgfr1 inactivation occurs in a cohort of cells within a clear boundary defined by the absence of Fgfr1 or a reduction of Spry4 and Mkp3 expression (Fig. 1L, Fig. 5H). A gene expression change confined within Fgfr1 cells will support that FGFR1 regulates this gene cell-autonomously. Third, direct comparison of gene expression between Fgfr1 and normal cells within the same limb bud has increased our ability to detect subtle changes.

To address expression regulation in the context of Shhcre:Fgfr1 limb buds, we first established Shhcre;Fgfr1 limb buds as a proper control. This is important because Shhcre is generated by insertion of Cre into the Shh-coding region. Thus, only one wild-type copy of Shh remains in Shhcre;Fgfr1 (Shhcre/+;Fgfr1co/co) mutants. We found that the Shhcre/+;Fgfr1+/- control limb buds at E10.75 show robust Shh expression, despite having only one wild-type Shh allele and one wild-type Fgfr1 allele (Fig. 5C). By contrast, in E10.75 Shhcre;Fgfr1 mutant limb buds, Shh expression is reduced to a punctate pattern (Fig. 5D), demonstrating that Fgfr1 cell-autonomously regulates Shh expression at the RNA level.

We noted that Shh expression is more reduced distally than proximally (asterisk in Fig. 5D). There is evidence that in a normal limb bud, endogenous Shh is expressed at a higher level distally than proximally (Dr Cliff Tabin, personal communication). Based on this, we favor the explanation that in Shhcre:Fgfr1 limb buds, the PD difference in Shh inactivation is a result of differential Shh activity, and hence differential Fgfr1 inactivation. Consistent with this, the expression patterns of Fgfr1 and Mkp3 at E10.75 (Fig. 5G,H; data not shown) indicate that FGF signal reception is efficiently reduced in the distal two-thirds, while it remains in the proximal one-third of the Shh active domain (compare Fig. 5H with 5B). At later stages, Shh expression is progressively more reduced in Shhcre;Fgfr1 limb buds, and eventually absent at E11.25, a time when it is still expressed in control limb buds (data not shown). To investigate factors that may mediate Fgfr1 regulation of Shh, we examined Hand2 (previously known as dHAND) expression, as it is necessary and sufficient to induce Shh expression (Charite et al., 2000). Hand2 expression does not change in Shhcre;Fgfr1 limb buds (data not shown), suggesting that HAND2 may act upstream of FGFR1, or that FGFR1 and HAND2 regulate Shh expression in parallel pathways.

As an example of non-cell-autonomous regulation, reduction of Ptc1 expression is not restricted to the Fgfr1 domain, consistent with the idea that FGFR1 regulates Ptc1 expression.
through secreted SHH (Fig. 5E,F). Previous studies show that Fgf10 expression in the mesenchyme is dependent on AER-FGF signaling (Boulet et al., 2004; Ohuchi et al., 1997). However, Fgf10 expression is unaltered in Shhcre;Fgfr1 limb buds (data not shown), suggesting that FGFR1 is not essential for Fgf10 expression. Of the three Bmp genes investigated above, Bmp2 expression remains normal, while Bmp4 and Bmp7 expression is reduced only within Fgfr1<sup>-/-</sup> cells, suggesting cell-autonomous regulation (Fig. 5I,J; data not shown).

The utility of Shhcre;Fgfr1 mutant limb buds in gene expression studies is best demonstrated by data on Hoxa13 and Hoxd13. In E11.5 Shhcre;Fgfr1 limb buds, we consistently detected reduced Hoxd13 expression in a wedge of cells in the anterior region of the Fgfr1<sup>−/−</sup> domain (n=5/5) (Fig. 5K,L). This suggests that FGFR1 cell-autonomously regulates the expression of Hoxd13, and that in the posterior Fgfr1<sup>−/−</sup> domain, its function may be redundant with other regulators of Hox expression. Interestingly, the expression of Hoxa13 is unaltered in Shhcre;Fgfr1 limb buds (data not shown), in contrast to our finding in Tcre;Fgfr1 limb buds. These data together demonstrate that the analysis of Shhcre;Fgfr1 limb buds has led to novel findings in FGF regulation of gene expression.

**Inactivating Fgfr1 in the Shh-expressing domain leads to absence of one digit**

In all forelimbs and hindlimbs of Shhcre;Fgfr1 mutants examined at E18.5 (n=6 embryos), the stylopod and zeugopod are normal in size, but the autopod is missing one digit (Fig. 6). Control (Shh<sup>cre/+;Fgfr1<sup>co/+</sup></sup>) limbs display a normal skeletal pattern, suggesting that the phenotype we observed in the mutant is not a result of loss of a single allele of Shh and Fgfr1.

The absence of a digit could be due to reduced growth or a defect in AP patterning. We uncovered evidence to support the latter hypothesis. In E11.5 Shhcre;Fgfr1 hindlimb buds, while limb bud size remains normal, only four condensations are detected by Sox9 expression (Fig. 7A,B). Compared with wild type, the two middle condensations in the mutant are each wider and farther apart than any of the wild-type digit pairs. The differences are more pronounced in E11.5 forelimb buds (Fig. 7C,D). These observations suggest that when starting with a mesenchymal field of equivalent size, the presence or absence of FGFR1 function can lead to a difference in the number of digits placed.

It is interesting that in Shhcre;Fgfr1 mutant only one digit is affected, as Shh<sup>cre</sup> is capable of inactivating target gene function in prospective digits 4, 5 and part of digit 3 (Harfe et al., 2004). Although limited by lack of digit-specific molecular markers, both condensation as well as skeletal pattern data support the conclusion that digit 3 is absent. In an E11.5 wild-type forelimb bud, each of the condensations exhibits a characteristic morphology.
In limb development

(Fig. 7C). In a Shhcre;Fgfr1 forelimb bud at the same stage, morphology as well as position of the four condensations indicate that digits 1, 2, 4 and 5 condensations are present, while digit 3 condensation is not (Fig. 7D). Examination of E18.5 skeletal preparations show that all mutants analyzed exhibit the same phenotype (n=6 skeletons). Based on phalanx number/shape, digit length and their metacarpal/metatarsal articulation with the carpals/tarsals, we determined that digits 1, 2 and 5 are present in the forelimb (Fig. 6A,B) and digits 1, 4 and 5 are present in the hindlimb (Fig. 6C,D). Thus, the combined evidence is consistent with the conclusion that digit 3 is absent in Shhcre;Fgfr1 mutant limbs.

Inactivating Fgfr1 affects Shh lineage

The deduction that digit 3 is absent in Shhcre;Fgfr1 mutant led us to investigate the mechanism behind this defect. Two recent studies proposed that the Shh-expressing cell lineage and the SHH-responsive cell lineage are important for the identities of digits 2-5 (Ahn and Joyner, 2004; Harfe et al., 2004). In particular, digit 3 forms at the anterior boundary of the Shh-expressing lineage, and is proposed to require the participation of Shh-expressing cells, as well as the influence of secreted SHH signaling (Harfe et al., 2004). We found that a change in the Shh-expressing lineage may account for the digit defect in the Shhcre;Fgfr1 mutant.

In an E11.75 forelimb bud comparing β-gal staining and Sox9 expression at an equivalent stage, the Shh-expressing lineage spans two and one-half digits, similar to wild type (Fig. 7E,F). However, the percentage of lacZ-expressing cells within the anterior portion of the lineage domain is significantly reduced in the mutant compared with normal. The cause of this reduction is not clear as neither cell death nor cell proliferation differences were detected (data not shown). The lineage reduction phenotype is confirmed at E12.5 when digits are apparent (Fig. 7G,H). Based on the hypothesized cellular and molecular requirements for digit identities (Harfe et al., 2004), we propose that at the anterior boundary of the Shh-expressing lineage in the Shhcre;Fgfr1 limb buds, a reduction in lineage contribution results in a local environment sufficient for the formation of digit 2 but not digit 3.

Discussion

In this study, we generated two mutants in which Fgfr1 is inactivated in a different temporal and spatial manner. Analyses of these mutants reveal multiple aspects of FGFR1 function in limb outgrowth and patterning (Fig. 8).
impacts limb skeletal formation in three phases (Fig. 8). In the early phase, FGFR1 is required for elongating the nascent limb bud along the PD axis and restricting it along the other two axes. In the middle phase, FGFR1 is required for mesenchymal cell survival. In the late phase, FGFR1 is required for autopod patterning by influencing digit placement and identity. The skeletal defect in Tcre;Fgfr1 mutant is probably due to a combined cellular deficiency in all three phases of limb development, while loss of one digit in Shhcre;Fgfr1 limbs is due to lack of FGFR1 function in the late phase.

At the molecular level, the reduced expression of several key patterning molecules, including Shh, Hoxa13 and Hoxd13, in the two mutants may explain their zeugopod and autopod defects. Certain Hoxa13/d13 homozygous/heterozygous combination mutants exhibit reduction of digit number and size, similar to that of Tcre;Fgfr1 mutant (Fromental-Ramain et al., 1996). Although not fully characterized, it is worth noting that the Tcre;Fgfr1 hindlimb skeletal phenotype closely resembles that of the Shh+/– mutant hindlimb (Fig. 2D) (Chiang et al., 2001; Kraus et al., 2001). As Shh expression is drastically reduced in the Tcre;Fgfr1 hindlimb buds (data not show), the phenotypic similarity is suggestive of a causal relationship. Furthermore, as discussed in more detail below, the lack of digit 3 in Shhcre;Fgfr1 mutant is probably due to the reduction of Shh expression in these limb buds. We note that Tcre;Fgfr1 mutant exhibits stylopod reduction that is not observed in either Shh+/– or 5′-Hox mutants. This suggests that there are additional factors mediating FGFR1 function.

**FGFR1 restricts cell number and influences the dimensions of a nascent limb bud**

The earliest role of FGFR1 in limb bud development is revealed by our analyses of Tcre;Fgfr1 mutant limb buds. These limb buds initiate normally, but shortly afterwards are misshapen and contain an increased number of cells. Using phosphorylated-Histone staining, we failed to detect any difference in cell proliferation that would account for the cell number increase, although it should be stated that this or any other available methods to detect cell proliferation differences are limited in sensitivity. An alternative explanation for both cell number and limb bud shape defects is that FGFR1...
signaling serves as a permissive cue for changes in cell adhesion, so that only a selected LPM population constitutes a limb bud with set dimensions. This hypothesis is put forth based on previous evidence that FGFR1 influences cell adhesion properties during gastrulation and limb bud formation (Ciruna and Rossant, 2001; Ciruna et al., 1997; Deng et al., 1997; Saxton et al., 2000). For example, in ES cell-derived chimera embryos, it was observed that Fgfr1-/- cells accumulate at the base of the limb bud while wild-type cells advance into the distal bud. At first glance, this predicts there would be fewer cells in Tcre:Fgfr1 nascent limb buds, in contrast to our observation. However, it should be noted that in Tcre:Fgfr1 embryos, all LPM cells in the vicinity of the prospective limb bud region are mutant for Fgfr1, unlike the situation in the ES-cell chimera where mutant cells intermingle with wild-type cells. When all LPM cells are equally compromised in adhesion strength, it is conceivable that by way of stochastic competition more cells from a wider region of the LPM would enter the limb bud. A similar scenario has been documented regarding FGFR1 function in notochord formation. ES cell chimera embryos composed of Fgfr1 mutant and wild-type cells show that the Fgfr1-/- cells are underrepresented in the chimeric notochord (Ciruna et al., 1997). Counterintuitively, a clearly expanded notochord is observed in the Fgfr1-null mutant (Deng et al., 1994; Yamaguchi et al., 1994). These seemingly contradictory data present a challenge to understand the precise cellular and molecular mechanisms underlying limb bud establishment. We found here that FGFR1 functions in this process to promote limb PD outgrowth, while restricting the dimensions of the other two axes. In addition, it serves an unexpected role in limiting cell number in a nascent limb bud.

**Fgfr1 expressed in LBM is essential for cell survival**

Similar excess cell death phenotypes are observed in Tcre:Fgfr1 mutant and in AER-Fgf mutants (Boulet et al., 2004; Lewandoski et al., 2000; Sun et al., 2002), suggesting that AER-FGF signaling mediated by FGFR1 is essential for mesenchymal cell survival. In a wild-type limb bud, limited cell death is observed in the proximal mesenchyme (Fig. 3O), in agreement with previous findings (Dawd and Hinchcliffe, 1971; Milaire and Rooze, 1983). We hypothesize that these cells die because they are out of the range of AER-FGF signaling. This is supported by several lines of evidence. First, beads soaked in FGF protein can rescue cell death following AER removal, suggesting that FGF can maintain LBM cell survival (Fallon et al., 1994). Second, secreted FGFs exhibit a limited range, possibly as a result of endocytosis and subsequent degradation in lysosomes (Scholpp and Brand, 2004). Third, using phosphorylated-ERK as an indicator of FGF signal activation, it has been shown that only cells in the distal mesenchyme of the limb bud are under the influence of FGF (Corson et al., 2003). This is confirmed by the expression patterns of Spry genes and Mkp3, which are transcriptionally regulated by FGF signaling (Eblaghie et al., 2003; Kawakami et al., 2003; Minowada et al., 1999). Consistent with our hypothesis, the expression pattern of Spry4 complements the observed cell death domain in normal limb buds (compare Fig. 3S with 3O). This limited range of FGF pathway activation is likely due to restricted diffusion of the ligands, as there is no evidence for a high-distal/low-proximal gradient in the expression of Fgfr1 and Fgfr2 in LBM cells (Orr-Urtreger et al., 1991; Peters et al., 1992; Yamaguchi et al., 1992).

Based on our hypothesis, we would argue that the increase in proximal cell death observed in Tcre:Fgfr1 and AER-Fgf mutant limb buds is due to reduced FGF reception and reduced FGF signaling range, respectively, leading to fewer proximal cells being protected from cell death compared with wild type. This is supported by the reduced domain of Spry2,4 and Mkp3 expression in these mutants (Fig. 3T; X.S., unpublished). Despite reduction, these indicators of FGF signaling are still expressed in the distal mesenchymal cells of Tcre:Fgfr1 limb buds (Fig. 3T; data not shown). This may explain why cell death in Tcre:Fgfr1 limb buds is not detected in the distal mesenchyme, unlike the situation following AER removal (Dudley et al., 2002; Rowe et al., 1982). We speculate that excess cell death in the Tcre:Fgfr1 limb buds contributes to the later reduction of limb skeleton, in particular the proximal elements.

**FGFR1 influences digit number and identity**

In both Tcre:Fgfr1 and Shhcre:Fgfr1 mutants, digit(s) are missing. However, we propose that the mechanism leading to the loss of digits is different in the two mutants. In the Tcre:Fgfr1 mutant, a reduction in progenitor cell number is probably the main cause, as evidenced by reduced limb bud size prior to digit condensation (Fig. 4). By contrast, in the Shhcre:Fgfr1 mutant, limb bud size remains normal when only four instead of five digit condensations are observed (Fig. 7A,B). This suggests that FGFR1 influences the selection of cells from the mesenchyme that constitute digit condensations.

We propose that this influence is achieved through FGFR1 regulation of Shh expression. In the Shhcre:Fgfr1 mutant, the observed downregulation of Shh RNA level would presumably result in reduced SHH production. It has been shown that a reduction in SHH production/distribution leads to loss of digits (Lewis et al., 2001).

We propose that at a later stage, FGFR1 also influences the determination of digit identity by regulating Shh expression. Recent studies of Shh-expressing and SHH-responsive cell lineages suggest that these are key parameters in the hypothesized rules of digit determination (Ahn and Joyner, 2004; Harfe et al., 2004). As discussed at the end of the Results section, based on these rules, the observed reduction of Shh-expressing lineage in Shhcre:Fgfr1 limb buds may be responsible for the failure to form a normal digit 3 in this mutant.

**Fgfr1 is required for the expression of key genes in limb bud patterning**

The Shhcre:Fgfr1 mutant is the first mutant in the FGF signaling pathway that offers a rigorous setting to test FGF regulation of gene expression during limb development. We report unequivocal evidence that FGF signaling regulates Shh at the RNA level, providing genetic support for the Fgfr/Shh feedback loop (Lauffer et al., 1994; Niswander et al., 1994). In addition, this mutant yields new data on whether FGFR1 regulates gene expression in a cell-autonomous manner. For example, as it was shown that SHH regulates the expression of Bmp genes (Lauffer et al., 1994; Yang et al., 1997), it is reasonable to hypothesize that FGFR1 regulates Bmp expression non cell-autonomously through the regulation of
Shh by FGFR1. However, in Shhcre;Fgfr1 limb buds, the sharp boundary between Bmp4-expressing and non-expressing cells corresponds well with the anterior boundary of Fgfr1 inactivation (arrows in Fig. S5H,J), suggesting that FGFR1 cell-autonomously regulates Bmp4 expression, instead of acting through secreted SHH.

The effectiveness of using Shhcre;Fgfr1 limb buds to assay gene expression is also demonstrated by our expression analysis of the paralogous group Hoxa13 and Hoxd13. Both genes are downregulated in Tcre;Fgfr1 limb buds (Fig. 4I-L), while only Hoxa13 is downregulated in Shhcre;Fgfr1 limb buds (Fig. 5L; data not shown). One possible explanation for this difference is that Fgfr1 may regulate Hoxa13 cell-autonomously and Hoxa13 non-cell-autonomously, Thus, the effect on Hoxa13 gene expression is evident only when Fgfr1 is inactivated in a larger domain in Tcre;Fgfr1 limb buds. Alternatively, Hoxa13 downregulation in Tcre;Fgfr1 limb buds could be largely due to reduction of the distal mesenchymal cell population that normally expresses Hoxa13. The combined gene expression data demonstrate that novel insights can be gained by revisiting FGF regulation of gene expression in Shhcre;Fgfr1 limb buds. As discussed above, Shh and 5′-Hox genes can mediate only a subset of FGFR1 function in limb skeleton formation. Our goal in the near future is to use Shhcre;Fgfr1 limb buds as a unique setting to identify other candidate mediators of FGF function in limb development.

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Supplementary material
Supplementary material for this article is available at http://dev.biologists.org/cgi/content/full/132/19/4235/DC1

References


Table S1. Total cell number in Tcre;Fgfr1 mutant forelimb buds compared with normal control

<table>
<thead>
<tr>
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<th>Tcre;Fgfr1</th>
<th>Normal</th>
<th>% change</th>
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<tbody>
<tr>
<td>E10.0</td>
<td>9.9±0.53×10^4 (n=4)</td>
<td>6.0±1.5×10^4 (n=6)</td>
<td>+65</td>
</tr>
<tr>
<td>E10.5</td>
<td>2.5±0.31×10^5 (n=6)</td>
<td>1.9±0.21×10^5 (n=6)</td>
<td>+31</td>
</tr>
<tr>
<td>E11.5</td>
<td>3.2±0.22×10^5 (n=6)</td>
<td>4.2±0.32×10^5 (n=6)</td>
<td>–26</td>
</tr>
</tbody>
</table>

The number (n) of single limb buds assayed for each genotype and stage is indicated. Embryos were dissected in Ca^{2+}/Mg^{2+}-free (CMF)-PBS and washed three times. Individual limb buds were incubated in 100 µl CMF-PBS for 30 minutes at 37°C. After incubation the ectoderm was peeled away from the mesenchyme. The mesenchyme was then dissociated by incubating in 100 µl of 0.5% trypsin (Invitrogen) with 1% pancreatin (Sigma) at 4°C for 20 minutes followed by 15 minutes at 37°C. After incubation 50 µl of fetal bovine serum (FBS) was added to the mixture. Limb bud pieces were collected by centrifuging at 3000 rpm for 30 seconds and washed three times each in 200 µl of DMEM with 10% FBS. After the final wash, 100 µl of DMEM was added and the limb bud was triturated until all tissue clumps disappeared. After centrifugation the pelleted cells were resuspended in 50 µl of DMEM and counted using a hemocytometer.