

FGFR-3 and FGFR-4 function cooperatively to direct alveogenesis in the murine lung

Michael Weinstein¹, Xiaoling Xu¹, Kyoji Ohyama² and Chu-Xia Deng^{1,*}

¹Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Diseases, 10/9N105, 10 Center Drive, National Institutes of Health, Bethesda, MD, 20892, USA

²Department of Anatomy, School of Medicine, Keio University, 35 Shinanomachi, Shinjuku-ku, Tokyo 160, Japan

*Author for correspondence (e-mail: ChuxiaD@bdg10.niddk.nih.gov)

Accepted 25 June; published on WWW 25 August 1998

SUMMARY

Mammalian lungs begin as an outpocket of the foregut, and depend on multiple stages of branching morphogenesis and alveogenesis to reach their final form. An examination of fgf receptor gene expression indicated that all four receptors (*fgfr-1* to *fgfr-4*) are expressed in postnatal lungs at varying levels. We show that mice homozygous for a targeted mutation of *fgfr-4* exhibited no overt abnormalities in the lungs or any other organ. However, mice doubly homozygous for disruptions of the *fgfr-3* and *fgfr-4* genes display novel phenotypes not present in either single mutant, which include pronounced dwarfism and lung abnormalities. Lungs of *fgfr-3*^{-/-}*fgfr-4*^{-/-} animals,

which are normal at birth, are completely blocked in alveogenesis and do not form secondary septae to delimit alveoli. Consequently, air spaces in the lung are expanded and no alveoli can be seen. The mutant lungs failed to downregulate postnatal elastin deposition despite their normal levels of surfactant expression and cell proliferation. These data revealed a cooperative function of FGFR-3 and FGFR-4 to promote the formation of alveoli during postnatal lung development.

Key words: FGFR-3, FGFR-4, Receptor cooperativity, Lung development, Secondary septation, Alveogenesis

INTRODUCTION

Lung development begins with the ventral budding of the lung primordium from the foregut. The lung bud divides into saccules, which further divide dichotomously and invade the surrounding mesenchyme. Saccule division results in the formation of the bronchial tree, while epithelial differentiation and formation of the air-blood barrier occur subsequently. The air spaces that are formed are expanded into prealveolar saccules, which are further subdivided into alveoli by secondary septation, a process that occurs largely after birth and increases the surface area of the lung manifold. These septae are remodeled to yield the mature alveoli of the lung (Burri, 1991).

FGFs are a family of 15 low molecular weight polypeptide growth factors, which function in a variety of ways during normal development and homeostasis (McWhirter et al., 1997; Smallwood et al., 1996; Verdier et al., 1997; Yamasaki et al., 1996). Gene disruption experiments in mice suggest that *fgf-3* (*int-2*) functions in patterning the inner ear and the caudal neural tube (Mansour et al., 1993), *fgf-4* is essential for embryogenesis at postimplantation stages (Feldman et al., 1995), *fgf-5* regulates hair follicle growth (Hebert et al., 1994), *fgf-7* (*kfg*) functions during the development of the integument (Guo et al., 1996), and *fgf-6* is necessary for muscle regeneration (Floss et al., 1997). During embryogenesis FGF-8 and FGF-10 have been reported to promote limb bud outgrowth (Crossley et al., 1996; Ohuchi

et al., 1997; Vogel et al., 1996) and FGF-8 has been implicated in the formation of the midbrain/hindbrain boundary (Crossley et al., 1996), while FGF-4 is thought to mediate the function of the zone of polarizing activity to pattern the limb bud (Niswander et al., 1994). Indeed topical application of exogenous FGFs are sufficient to induce ectopic limb buds in the interlimb region of chicken embryos (Cohn et al., 1995).

The actions of FGFs are mediated by the FGFRs, a family of four single pass transmembrane receptors with ligand-induced tyrosine kinase activity. All four receptors share a similar protein structure, consisting of three Immunoglobulin-like (Ig) domains on the extracellular side of the cell membrane linked to a split tyrosine kinase domain in the cytoplasm. The Ig domains comprise the ligand-binding site of the receptors and are subject to alternative splicing, creating isoforms with unique FGF-binding specificities (reviewed in Johnson and Williams, 1993).

The genes encoding all four FGFRs have been disrupted in mice. *fgfr-1* is required during embryogenesis and animals lacking it show globally decreased levels of cell proliferation. They suffer arrested development before or during gastrulation and exhibit an increased amount of axial mesoderm, at the expense of paraxial and lateral mesoderm (Deng et al., 1994; Yamaguchi et al., 1994). More recently chimeric mouse embryos formed using FGFR-1 null ES cells revealed a role of FGFR-1 in multiple developmental processes, including mesodermal cell migration, posterior neural tube development,

and the growth and patterning of the limb buds (Ciruna et al., 1997; Deng et al., 1997).

fgfr-2 is also required during embryogenesis. A null mutation results in embryonic lethality at day 4 of development, as the embryo no longer implants properly in the uterus (Arman et al., 1998), while a hypomorphic allele of *fgfr-2* generated in our laboratory reveals essential functions of FGFR-2 in placental and limb bud initiation (Xu et al., 1998). *fgfr-3* mutants are viable and fertile, but they suffer skeletal dysplasias due to the increased growth of the long bones. FGFR-3 appears to mediate an inhibitory signal, restraining the growth of proliferating chondrocytes during endochondral ossification (Colvin et al., 1996; Deng et al., 1996). This phenotype is apparently the opposite of humans with *fgfr-3*-dependent skeletal dysplasias (reviewed in Muenke and Schell, 1995).

The *fgfr-4* gene is located on chromosome 13 in mice and 5q33-qter in humans (Avraham et al., 1994), and is unusual in that it does not undergo the alternative splicing described for the other receptors (Vainikka et al., 1992). FGFR-4 binds strongly to acidic FGF, basic FGF and FGF-4, FGF-6, FGF-8 and FGF-9, and it also binds FGF-3 and FGF-5 with less affinity (Goldfarb, 1996). FGFR-4 associates with an 85 kilodalton (kDa) serine kinase, which it can activate in a process that is dependent on ligand binding but independent of receptor tyrosine phosphorylation (Vainikka et al., 1996). During embryonic days 8.5-9.5 (E8.5-9.5), *fgfr-4* transcripts can be found in the endodermal compartments of the gut and yolk sac and in the myotomal compartment of the somites. By E14.5, *fgfr-4* expression is seen in the developing skeletal muscles, a number of endodermally derived tissues including liver, lung and pancreas, and the adrenal cortex and kidney (Stark et al., 1991). At E16.5, *fgfr-4* is also found in the ventricular zone and cortex of the murine brain (Ozawa et al., 1996). *fgfr-4* expression has also been seen in a number of mammary carcinomas, suggesting an involvement in breast cancer (Jaakkola et al., 1993).

We have examined the expression of all four FGFRs in the postnatal murine lung and found that the FGFRs are all expressed postnatally. To examine its role during lung development, we have disrupted *fgfr-4* in the murine germline. *fgfr-4*^{-/-} animals do not exhibit any obvious abnormalities, suggesting that *fgfr-4* is largely dispensable for normal development. However, we have found that animals doubly homozygous for targeted disruptions of *fgfr-3* and *fgfr-4* display a unique phenotype not found in either mutant alone. We present evidence that FGFR-3 and FGFR-4 function cooperatively to mediate events crucial for development of the postnatal lung.

MATERIALS AND METHODS

Targeting vector

Recombinant phages containing genomic DNA of the *fgfr-4* locus were isolated from a 129 mouse library (Stratagene). To construct the *fgfr-4* targeting vector, a 2.5 kilobase (kb) *KpnI* fragment that is 3' to the *KpnI* site in exon 6 of the *fgfr-4* gene was subcloned into the *KpnI* site of pPNT (Tybulewicz et al., 1991). The resulting construct was cleaved with *XhoI* and *NotI*, followed by insertion of a 5 kb *KpnI*-*NotI* fragment (the *NotI* site is from the polylinker of the phage vector). The finished construct, pFGFR-4neo, is shown in Fig. 3A.

Homologous recombination in ES cells and generation of germline chimeras

TC1 ES cells were transfected with *NotI*-digested pFGFR-4neo and

selected with G418 and FIAU. The culture, electroporation and selection of TC1 cells was carried out as described (Deng et al., 1994). ES cell colonies that were resistant to both G418 and FIAU were analyzed by Southern blotting for homologous recombination events within the *fgfr-4* locus. Genomic DNAs from these clones and the parental TC1 cell line were digested with *SsrI*, and then probed with the 5' probe specific to the *fgfr-4* sequence shown in Fig. 3A.

ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts to obtain germline transmission. The injected blastocysts were implanted into the uteri of pseudopregnant Swiss Webster (Taconic) foster mothers and allowed to develop to term. Male chimeras (identified by the presence of agouti coat color) were mated with NIH Black Swiss females (Taconic). Germline transmission was confirmed by agouti coat color in the F₁ animals and all agouti offspring were tested for the presence of the mutant *fgfr-4* allele by Southern analysis or PCR.

Genotype analysis

Genotypes were determined by Southern blotting or PCR. For PCR analysis, the wild-type *fgfr-4* allele was detected by using a 5' oligonucleotide (5'-ACCAACTGGAGCCTGGT-3') and a 3' oligonucleotide (5'-AGGAGATAGCTGTAGCGAATGC-3'). This primer pair flanks the *PGKneo* insertion site and amplifies a 106 bp fragment from the wild-type *fgfr-4* gene. DNA was also amplified using a primer that is specific to the *neo* gene (5'-ATCGCCTTCTATCGCCTTCTTGACGAGTTC-3') to detect the mutant *fgfr-4* allele (Deng et al., 1994). In this case, a 431 bp fragment is detected in mice heterozygous or homozygous for the disrupted *fgfr-4* allele, while no signal can be detected in wild-type mice.

Generation of FGFR-3 and FGFR-4 double homozygous mice

The *fgfr-3* mutation was genotyped by PCR using an *fgfr-3* 5' oligonucleotide (5'-GGCTCCTTATTGGACTCGC-3') and a 3' oligonucleotide (5'-TCGGAGGGTACCACACTTTC-3'). This primer pair flanks the *PGKneo* insertion site and amplifies a 308 bp fragment in wild-type and *fgfr-3*^{+/-} mice. DNA was also amplified with the *neo* primer shown earlier, which in combination with the 3' oligonucleotide amplifies a 300 bp fragment in *fgfr-3*^{+/-} and *fgfr-3*^{-/-} animals. The *fgfr-4* mutation was maintained on a mixed 129/Black Swiss background. To generate double mutants, *fgfr-4*^{-/-} animals were bred with *fgfr-3*^{-/-} animals (Deng et al., 1996) and the resulting double heterozygotes were intercrossed. Once lines were established, double mutants were generated by interbreeding *fgfr-3*^{+/-}/*fgfr-4*^{-/-} animals.

Expression analysis

RNA was isolated from the livers and lungs of *fgfr-4*^{+/-} and *fgfr-4*^{-/-} animals using RNA-Stat (Tel Test, Friendswood, TX), and poly(A)-enriched RNA was purified using the mRNA purification kit of Pharmacia, both according to the manufacturers' protocols. mRNA was fractionated and blotted according to standard procedures and probed with a 1.5 kb *ApaI* fragment of the *fgfr-4* cDNA (a gift of Dr Andy McMahon), which includes the Ig domains and the 5' untranslated sequence of the *fgfr-4* gene. Multiple tissue northern blots, purchased from Clontech, were also probed with sequences derived from all four FGFRs (Deng et al., 1996). Total lung RNA was similarly fractionated and probed with elastin sequences (a kind gift of Rich Pierce) and L32.

RT-PCR analysis

Lung RNA was isolated using RNA-Stat (Tel Test, Friendswood, TX), and reverse transcription was carried out using the first-strand cDNA synthesis kit of Boehringer Mannheim according to the manufacturer's directions. cDNAs were then amplified using primers specific to elastin (5'-TTAGGAGTCTCGACAGGTGCT-3' and 5'-CAGCTCCATACTTAGCAGCCT-3') or glyceraldehyde phosphate dehydrogenase, (GAPDH; 5'-ACAGCCGCATCTTCTTGTGC-3' and

5'-TTTGATGTTAGTGGGGTCTCGC-3'). PCR products were electrophoresed through agarose gels and visualized by ethidium bromide staining. A 1:10 serial dilution series was created starting with 100 ng of subcloned elastin sequences. This was used as a template for PCR utilizing the elastin primers above, to ensure that the signal observed was within the linear range of the potential response (Ozawa et al., 1996).

Histology and immunohistochemistry

Tissues were fixed overnight in 10% neutral buffered formalin, dehydrated through an ethanol series and embedded in paraffin using standard procedures. Sections were cut and stained with hematoxylin and eosin. Elastin-Van Geimsa staining was carried out by American Histolabs (Gaithersburg, MD). Antibodies for FGFR-3 and FGFR-4 were purchased from Santa Cruz and immunohistochemistry was carried out as described (Fukuda et al., 1997). SPB and SPC antibodies were a gift of Dr Jeffrey Whitsett and SPA antibodies were supplied by Dr Frank McCormack. Staining with these antibodies was monitored with a Vectastain Elite Kit, which was used according to the manufacturer's protocols. The smooth muscle actin antibody was a product of Vector labs and used according to the manufacturer's directions. 200 µCi of [³H]thymidine (Amersham) was injected intraperitoneally into P10 mice, which were killed after 2 hours. Incorporation was monitored by autoradiography of paraffin sections.

RESULTS

Expression of FGFRs during lung development

FGFRs are known to play an important role early in lung development, as a dominant negative *fgfr-2* gene expressed from a surfactant promoter results in an absence of branching morphogenesis (Peters et al., 1994). We were interested in the roles played by the various FGFRs and therefore scrutinized the expression of all four FGFR genes in a panel of adult tissues that included lung (Fig. 1). All four FGFRs are

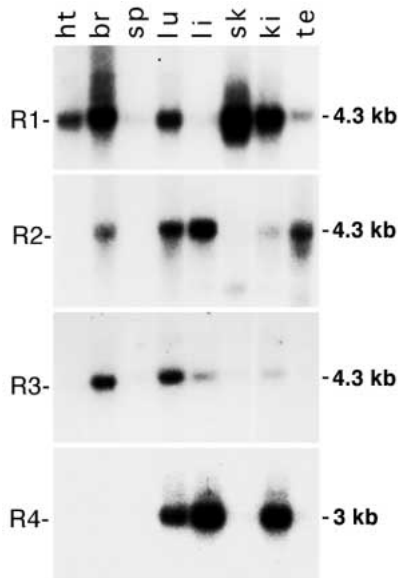


Fig. 1. FGF Receptor expression in various organs and tissues. Filters bearing mRNAs derived from multiple tissues (Clontech) were hybridized with sequences from the four FGFRs. ht, heart; br, brain, sp, spleen; lu, lung; li, liver; sk, skeletal muscle; ki, kidney; te, testis.

expressed in the lung at appreciable levels, in contrast to other tissues, such as heart and skeletal muscle, in which only *fgfr-1* is expressed, or the testes, which only produce *fgfr-1* and *fgfr-2* (Fig. 2A). The expression of FGFR-3 and FGFR-4 was examined in P10 lungs by immunohistochemistry (Fig. 2A,B, respectively). Ubiquitous staining of both was seen throughout the lung parenchyma.

Targeted disruption of the *fgfr-4* gene

fgfr-4 was disrupted by interrupting exon 6, which encodes the IgII domain, with the *PGKneo* gene. This is expected to create a null allele, as *fgfr-4* has not been shown to undergo the alternative splicing that characterizes the other members of the FGFR gene family and the IgII domain is common to all the isoforms of those receptor genes that are alternatively spliced. The targeting construct is shown in Fig. 3A. Disrupted *fgfr-4* alleles are detected by a 2 Kb increase in the 6 Kb *SstI* band (Fig. 3B). Homologous recombination at the *fgfr-4* locus was found in 17 out of 60 G418/FIAU doubly resistant ES clones analyzed.

Mice lacking *fgfr-4* develop normally

Two ES clones carrying the disrupted *fgfr-4* gene were injected into blastocysts and germline transmission was obtained from both. Mice heterozygous for the targeted mutation were produced and bred to give homozygous offspring. *fgfr-4*^{-/-}

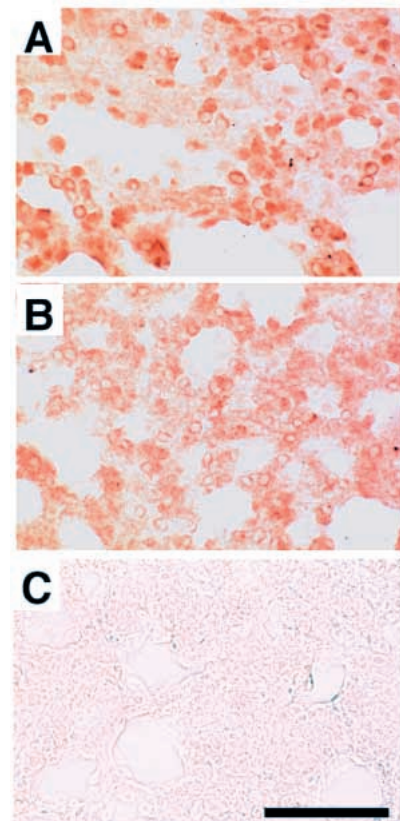


Fig. 2. Immunohistochemical staining is shown of lungs from a P10 wild-type animal stained with an antibody recognizing FGFR-3 (A) or FGFR-4 (B). Lungs from an *fgfr-4*^{-/-} animal were stained with an FGFR-4 antibody in C. Note the lack of any appreciable staining. Bar, 16 µm.

animals were born at the expected frequency and showed no gross abnormalities. They exhibited apparently normal growth, although they weighed 10% less than siblings at weaning (data not shown). Nevertheless they successfully reproduced with litter sizes indistinguishable from their wild-type counterparts. Indeed, *fgfr-4*^{-/-} animals over 1 year of age have not displayed any developmental irregularities. To ensure that the *fgfr-4* gene was truly disrupted in our animals, we probed northern blots of liver RNA for *fgfr-4*, where the gene has been shown to be transcribed (Stark et al. 1991; Fig. 2). *fgfr-4* transcripts were seen in the livers of *fgfr-4*^{+/-} mice, while their *fgfr-4*^{-/-} siblings showed no detectable expression (Fig. 3C). We have also probed the lungs of *fgfr-4*^{-/-} animals with an antibody to FGFR-4. Wild-type lungs exhibit abundant expression of FGFR-4 (Fig. 2B), while those from the homozygous animals are not detectably stained (Fig. 2C). These data suggest that we did indeed create a null mutation.

We conducted a histological analysis of organs and tissues from *fgfr-4*^{-/-} animals that express *fgfr-4* during embryogenesis (Stark et al., 1991) or adulthood (Fig. 1), but we did not find any abnormalities. Lungs isolated from E11.5 *fgfr-4*^{-/-} animals also responded normally to exogenously added FGFs in vitro (W. Cardoso, personal communication). Our finding of normal mice lacking *fgfr-4* gene expression suggested two possibilities, one of which is that *fgfr-4* is not essential for normal development. Numerous genes, thought to be essential for development, have been disrupted to yield apparently normal mice. However, *fgfr-4* belongs to a gene family with at least three other members. It is also possible that the functions of *fgfr-4* can be carried out by other FGFRs in its absence.

FGFR-3 and FGFR-4 function cooperatively

In order to test the latter possibility, we mated animals carrying the *fgfr-4* mutation to ones carrying a disrupted *fgfr-3* allele. *fgfr-3*^{-/-} animals are viable, but display a number of skeletal abnormalities due to an increased proliferation of proliferating chondrocytes at the growth plates of the long bones (Colvin et al., 1996; Deng et al., 1996). Animals doubly heterozygous for *fgfr-3* and *fgfr-4* mutations are indistinguishable from wild-type animals, as are *fgfr-3*^{+/-}*fgfr-4*^{-/-} mice. Moreover, *fgfr-3*^{-/-}*fgfr-4*^{+/-} animals were indistinguishable from their *fgfr-3*^{+/-}*fgfr-4*^{+/-} siblings (data not shown). However, *fgfr-3*^{-/-}*fgfr-4*^{-/-} animals, although viable, show defects not seen in either single mutant.

fgfr-3^{-/-}*fgfr-4*^{-/-} animals suffer growth retardation and a sickly appearance

The *fgfr-3*^{-/-}*fgfr-4*^{-/-} animals were much smaller than sibling controls, which included *fgfr-3*^{+/-}*fgfr-4*^{-/-} and *fgfr-3*^{+/+}*fgfr-4*^{-/-} mice (Fig. 4A). A measurement of the weights of 20 litters showed that the double mutants were 50% the size of siblings at weaning, despite the fact that they are indistinguishable from littermates at birth (data not shown). Growth curves indicate that the double mutants grew more slowly than their phenotypically normal siblings (Fig. 4A). Animals deficient in growth hormone display a similar growth phenotype, so we examined the level of growth hormone in double mutant pituitaries using an anti-growth hormone antibody. The double mutants were indistinguishable from controls (data not shown), suggesting that the growth defect is not due to a lack of growth hormone. However, there could be defects in the secretion or reception of the growth hormone signal.

fgfr-3^{-/-}*fgfr-4*^{-/-} mice were sickly and appeared dehydrated. They were also largely infertile, although a few animals produced progeny. In addition, there was a marked diminution in their vitality and the mutants usually died within the first few months of life (Fig. 4B). It is therefore possible that the growth defects are a secondary result of a more fundamental problem.

Double mutants exhibit lung defects

A histological analysis was conducted on the organs of double mutant mice to determine the cause of their apparent illness. Lungs isolated from *fgfr-3*^{-/-}*fgfr-4*^{-/-} animals were outwardly normal and dissections showed that the individual lobes were normal in shape and arrangement (data not shown). However, when examined histologically they exhibited an emphysematous appearance (Fig. 5G,H), and image analysis indicated that the air spaces in the double mutants were an average of three times larger than in the sibling controls. The architecture of the lung parenchyma was considerably simplified, and alveoli were not observable (Fig. 5I,J).

The lung phenotype seen in the *fgfr-3*^{-/-}*fgfr-4*^{-/-} animals is due to the failure of secondary septation

We examined lungs from animals at various ages to define the point at which the abnormalities ensued. At postnatal day 2 (P2), the lungs of double mutants were indistinguishable from those of their control siblings (Fig. 5A,B), with both displaying prealveolar saccules of approximately the same size. However, P9 lungs of mutants exhibited a clear difference from those of sibling controls. The sibling lung was undergoing the process of alveogenesis and could be seen to have already formed

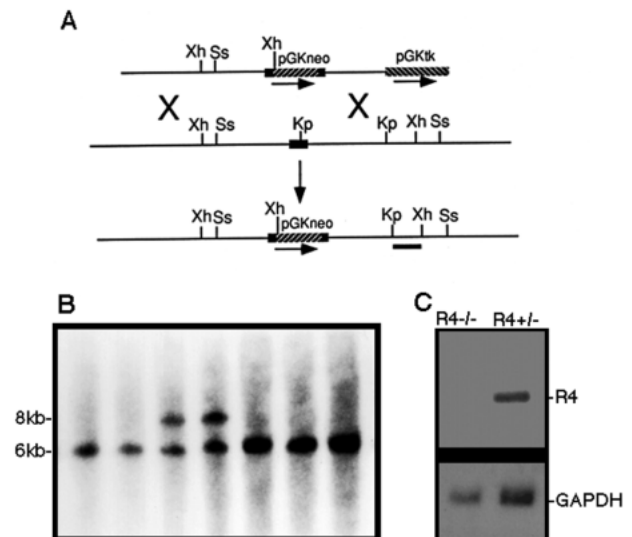


Fig. 3. Disruption of FGFR-4 by homologous recombination. (A) Structure of the targeting vector and the genomic locus. The targeting vector is shown at the top, the genomic DNA is in the middle, and the locus after homologous recombination is shown at the bottom. The black box is *fgfr-4* exon 6, and the striped boxes are regions derived from *Neo* and *TK*. Xh=*Xho*I, Ss=*Sst*I (*Sac*I), Kp=*Kpn*I. (B) Southern blot of DNA derived from embryonic stem cells after double selection. Approximately 30% of the cells examined had the expected shift of the *Sst*I fragment from 6 Kb to 8 Kb. (C) Northern blot of liver RNA derived from *fgfr-4*^{+/-} and *fgfr-4*^{-/-} animals probed for *fgfr-4* expression. The lack of R4 expression suggests the gene has indeed been disrupted.

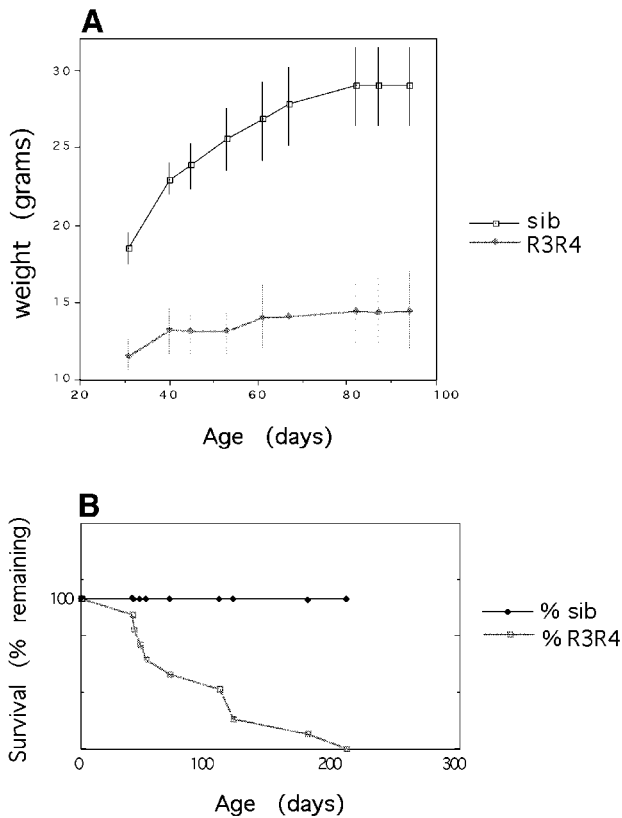


Fig. 4. *fgfr-3^{-/-}fgfr-4^{-/-}* animals grow more slowly and exhibit shorter life span than their aphenotypic littermates. (A) Weights were measured on 6 sibling and mutant animals and used to construct growth curves. (B) *fgfr-3^{-/-}fgfr-4^{-/-}* double mutants display reduced viability ($n=10$).

alveoli (arrows in Fig. 5C). Secondary septae are seen growing out from the walls of the prealveolar saccules (arrows in Fig. 5E) to delineate the alveoli, thus increasing the surface area available for gas exchange. But the double mutant lung retained its simplified architecture, in which the morphology was not much changed from that of the P2 animals (Fig. 5D,F). At this point, the animals themselves are clearly distinguishable, as the control siblings are growing more rapidly than the double mutants (data not shown). By P21, there were major differences between the lungs of double mutants and siblings. The sibling had elaborated many alveoli (Fig. 5G), shown in higher magnification in Fig. 5I. The mutant lungs remained relatively unchanged from those of P2 animals, and the walls of the mutant airways were smooth, unlike those of the phenotypically normal animals (Fig. 5J). We therefore believe that the lung defects seen in the *fgfr-3^{-/-}fgfr-4^{-/-}* mutant animals are due to a failure of secondary septation, which blocks the terminal steps of lung formation leading to an abnormal and immature lung.

Lungs of *fgfr-3^{-/-}fgfr-4^{-/-}* mutant animals are normal in some respects

Mutants lacking the growth factor PDGFA also exhibited a defect in alveogenesis and lacked staining for smooth muscle actin, suggesting a lack of smooth muscle myofibroblasts. We examined the lungs of *fgfr-3^{-/-}fgfr-4^{-/-}* animals and their phenotypically normal siblings with an antibody to smooth

muscle actin and found a comparable number of stained cells in each (Fig. 6A,B). We also inspected the surfactant complement of the mutant lungs by immunolabelling SPA and SPB, two of the major surfactant proteins in the lung, as well as pSPC, a precursor of another major surfactant. The data, an example of which is shown in Fig. 6C and D, indicated that the lungs from *fgfr-3^{-/-}fgfr-4^{-/-}* animals showed a normal number of cells producing these proteins, and the level of surfactant protein production was not markedly altered. The proliferation of cells in the lungs of double mutants was measured by incorporation of [³H]thymidine, because a decrease in cell proliferation could easily result in the observed phenotype. However, to our surprise this assay showed that cell proliferation was normal in the mutants' lungs during the process of alveogenesis (Fig. 6F,G). Approximately 10% of the cells in both P10 double mutant and sibling lungs were labelled.

Double mutant lungs fail to shut off elastin synthesis subsequent to alveogenesis

To define further the etiology of the double mutants' lung abnormalities, we stained histological sections for elastin fibers, which are known to be important for the architecture of the lung. Indeed, the *pdgfa* mutants eluded to earlier lack elastin fibers in the lung and have been reported to bear a superficial resemblance to the *fgfr-3^{-/-}fgfr-4^{-/-}* double mutants described here (Bostrom et al., 1996). Histochemical staining indicated that P5 *fgfr-3^{-/-}fgfr-4^{-/-}* animals had elastin fibers of normal appearance (Fig. 7A,B), suggesting that a failure of elastin deposition was not responsible for the defects seen. However, older animals exhibited a marked increase in elastin staining. For example, in the P60 lungs shown in Fig. 7C,D, the *fgfr-3^{-/-}fgfr-4^{-/-}* lungs exhibit abnormally large amounts of elastin (Fig. 7D) compared to sibling control animals (Fig. 7C). This increased elastin deposition became visible after alveogenesis, usually starting around P21 (data not shown).

Elastin synthesis is normally shut off following alveogenesis, with remodeling of the pulmonary extracellular matrix carried out largely by metalloelastases (Mariani et al., 1997). Before and during alveogenesis, elastin deposition appeared normal (Fig. 7A,B). To determine whether elastin synthesis was downregulated normally, we conducted an analysis of elastin expression in the lungs of double mutants and their siblings. We used lung cDNAs as templates for RT-PCR in an attempt to amplify elastin sequences. Our analysis indicates that elastin synthesis is easily detected in the sibling control and mutant lungs at the time of alveogenesis (P12, Fig. 7E). However, the signal subsequently disappears in the sibling and is no longer visible in the lungs of adult animals. On the contrary, the elastin message does not appear to be diminished in the lungs from the older *fgfr-3^{-/-}fgfr-4^{-/-}* mice and is at roughly the same level throughout postnatal lung development. Elastin synthesis appears normal during alveogenesis, as a similar level of tropoelastin message is detected in P10 *fgfr-3^{-/-}fgfr-4^{-/-}* mutants and their phenotypically normal siblings (Fig. 7F).

DISCUSSION

FGFR-4 is dispensable for normal development

We have disrupted the *fgfr-4* gene in the murine germline and found that mice lacking it develop without any obvious

abnormalities. It is interesting that *fgfr-4* is the only FGFR gene that does not seem to function during development and it is also the only one that does not undergo alternative splicing. Alternative splicing of the Ig domains creates receptors with different ligand specificities, and our laboratory has generated results suggesting that the various isoforms have unique functions. For example, the full-length isoform of FGFR-1 appears to be required uniquely for notochord regression (our unpublished observations), while the KGFR and BEK isoforms of FGFR-2 appear to function during mesenchymal-epithelial interactions in the developing limb bud (Xu et al., 1998). The

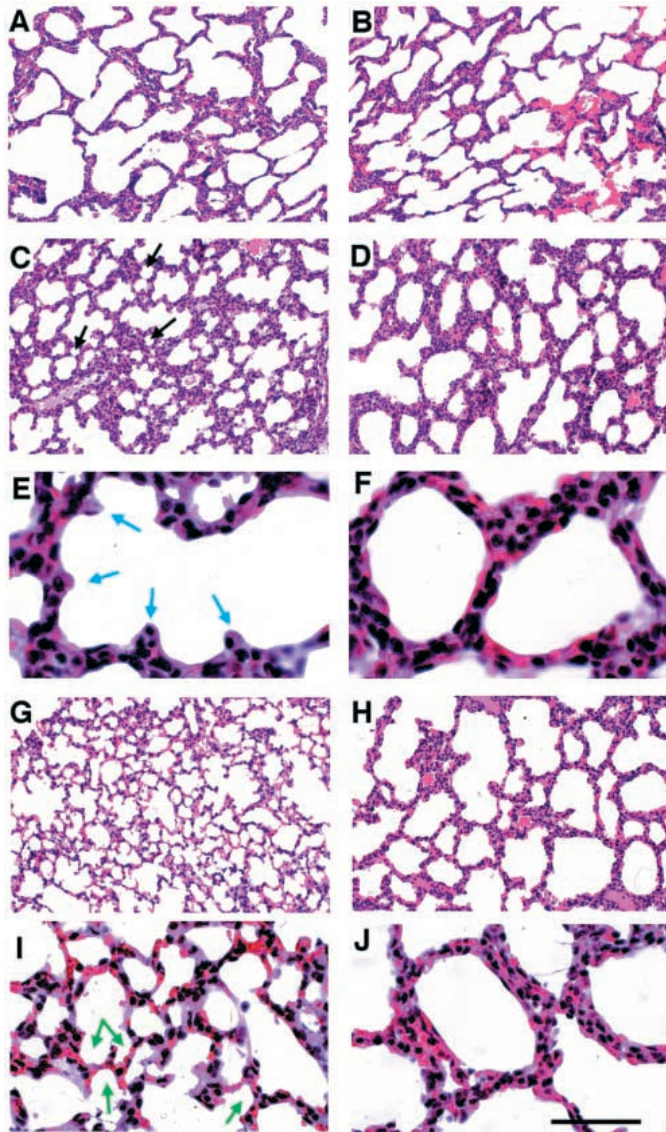


Fig. 5. *fgfr-3^{-/-}fgfr-4^{-/-}* animals do not carry out alveolar septation. Hematoxylin-Eosin stained lung sections from sibling controls (A,C,E,G,I) and *fgfr-3^{-/-}fgfr-4^{-/-}* animals (B,D,F,H,J). (A,B) P2. The mutants (B) are indistinguishable from siblings (A). (C-F) P9. Secondary septae are forming in the sibling (arrows in E), but not in the mutant (F). Arrows in C, alveoli. (G-J) P 21. The sibling lung (G,I) has formed numerous alveoli (arrows in I), while the mutant lung (H,J) still resembles that of a day 9 animal. Bar, 35 μ m in A-D, G and H, and 10 μ m in E, F, I and J.

lack of isoforms for FGFR-4 might suggest a more limited function during mammalian development.

Our results do not rule out a more subtle phenotype in the *fgfr-4^{-/-}* mutants. For example, mice homozygous for a disrupted vimentin allele, which are also largely normal, exhibit disruptions of the astrocyte cytoskeleton visible only with the electron microscope (Colucci-Guyon et al., 1994; Galou et al., 1996). Disruption of several other *fgf* genes has yielded relatively mild phenotypes (Guo et al., 1996; Hebert et al., 1994) and it may be that this is due to cooperativity between members of the *fgf* gene family. Cooperativity is a well-known phenomenon among highly homologous mammalian genes and it has been a widely reported phenomenon with respect to the *hox* genes. Knockouts of many murine *hox* genes result in mild skeletal distortions. However, when two knockout lines are interbred to generate double homozygotes at paralogous loci, entire skeletal elements are deleted. The *hox* genes appear to act in additive fashion, in that when a greater number of paralogs are deleted a more severe phenotype results (Chen and Capecchi, 1997; Davis et al., 1995).

We surmised that a lack of a developmental phenotype in the *fgfr-4^{-/-}* animals may be due to cooperativity among the different FGFRs. The four receptors have broadly overlapping ligand specificities and multiple FGFRs can be found in the same tissue as well. For instance, our data show that *fgfr-4* is expressed in the adult lung, liver and kidney. The liver shows expression of *fgfr-2* and *fgfr-3* as well, and both *fgfr-1* and *fgfr-2* are found in the kidney (Fig. 1). All four FGFRs are

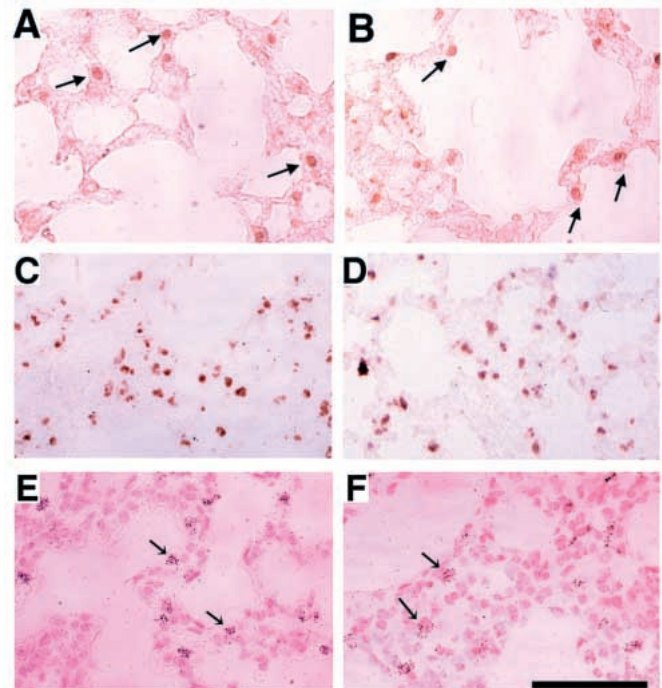


Fig. 6. Lungs of *fgfr-3^{-/-}fgfr-4^{-/-}* animals are normal in some respects. (A,C,E) *fgfr-3^{+/-}fgfr-4^{+/-}*. (B,D,F) *fgfr-3^{-/-}fgfr-4^{-/-}* (A,B) Immunolabelling of P15 lungs with an antibody to smooth muscle actin. (C,D) Immunolabelling of P60 lungs with an antibody against SPB. (E,F) P10 animals were injected with [³H]thymidine. Arrows point to positively labelled cells in A, B, E and F. Bar 14 μ m in A-D, and 35 μ m in E and F.

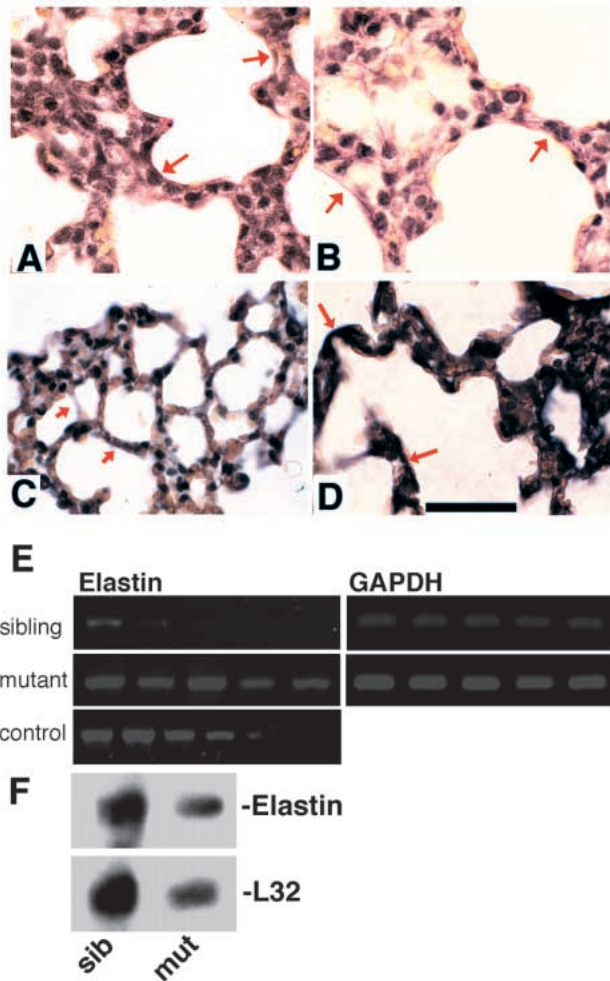


Fig. 7. Double mutant lungs exhibit increased levels of elastin deposition after alveogenesis. Elastin-Van Geimsa-stained lungs from (A,C) *fgfr-3^{+/}fgfr-4^{-/-}* and (B,D) *fgfr-3^{-/-}fgfr-4^{-/-}*. (A,B) P5; (C,D) P60. The arrows point to elastin fibers. (E) Elastin synthesis was examined by RT-PCR. cDNAs derived from postnatal lung RNAs were amplified with either elastin or GAPDH-specific primers. The ages of the animals used are shown at the top in days. The control PCR below used a 10-fold serial dilution series of elastin sequences amplified with the elastin primers. The amount of DNA used as template is shown at the top in nanograms. (F) RNAs from P10 sibling control (sib) and *fgfr-3^{-/-}fgfr-4^{-/-}* (mut) animals were probed with tropoelastin sequences. The blot was probed with L32 sequences as a loading control. Bar, 14 μ m.

expressed in the lung, where the FGFR-3 and FGFR-4 expression patterns overlap to a large degree (Fig. 2A,B).

FGFRs direct development of the lungs

The function of fibroblast growth factors and their receptors in the development of the respiratory system appears to be conserved and ancient. The *Drosophila melanogaster* FGF homolog *branchless* functions in the branching morphogenesis of the tracheal system (Sutherland et al., 1996), as does its receptor, *breathless* (Glazer and Shilo, 1991). Moreover, these genes function at different points during tracheal cell branching and migration (Lee et al., 1996), similar to what we and others have shown for their vertebrate homologues. In mammals, a

dominant negative FGFR has been shown to completely block the branching morphogenesis of the lung when expressed from a surfactant promoter (Peters et al., 1994). This branching is known to depend on epithelial-mesenchymal interactions, as lung buds deprived of mesenchyme fail to branch in vitro (Masters, 1976) unless provided with exogenous FGFs (Deterding et al., 1996). Functional evidence for the involvement of FGFR-2 comes from the overexpression of KGF (FGF7), which is a ligand for FGFR-2IIIb. When expressed from a surfactant promoter in transgenic animals (Simonet et al., 1995) or when applied to embryonic lungs in an in vitro culture system (Cardoso et al., 1997) KGF can cause abnormal cystic lung development. However, gene disruption experiments have been insufficient to resolve the role of FGFR-1 and FGFR-2, as animals deficient in both of these die well before the onset of lung development.

FGFR-3 and FGFR-4 are needed for alveolar septation of the postnatal lung

Analysis of knockout and transgenic animals has implicated numerous signaling molecules in lung development. For example, disruption of the *ttf-1* gene results in a complete absence of branching morphogenesis (Kimura et al., 1996), while loss of N-myc and the EGF receptor both result in a lung defect early in development due to a lack of cell proliferation (Kaartinen et al., 1995; Moens et al., 1992; Sibilia and Wagner, 1995). Loss of TGF β also results in aberrant lung development, while overexpression of sonic hedgehog (*shh*) and BMP-4 can disrupt branching morphogenesis (Bellusci et al., 1996, 1997), as can mutation of the *shh* target *gli-3* (Grindley et al., 1997).

Notably, all of the above-mentioned factors affect relatively early steps in lung formation. The lung abnormality caused by the loss of FGFR-3 and FGFR-4 is unique in that it very specifically affects a late step of pulmonary development, i.e. alveogenesis. Our results show that all four receptors are in the adult lung (Fig. 1), and we have detected their presence in the lung before, during and after alveogenesis by RT-PCR (Fig. 1 and data not shown). Our results are in agreement with other studies showing FGFR-3 and FGFR-4 expression in the lung (Chellaiah et al., 1994; Stark et al., 1991). We have not ruled out a function for FGFR-1 and FGFR-2 during late stages of lung development, but elucidating these will require further study.

At birth rodent lungs are subdivided into prealveolar saccules, which are composed of two distinct capillary beds, with an elastin- and collagen-poor interstitium. One of these capillary beds invaginates to form a secondary septum, which contains two capillary layers and an elastin fiber at its tip (Burri, 1991). Elastin fibers, based on their position in the septae, are thought to provide mechanical support during this process. Indeed, the lungs of mice deficient in *pdgfa* contain no elastin, and they also fail to carry out alveogenesis (Bostrom et al., 1996). However, the phenotype described here would appear to be more specific to the septation process than the defect seen in the *pdgfa^{-/-}* animals, because the latter lack alveolar myofibroblasts, undergo collapse of the lung parenchyma within a couple of weeks of birth and die soon thereafter (Bostrom et al., 1996). The *fgfr-3/fgfr-4* phenotype is considerably milder, as alveolar myofibroblasts are present, the lungs are functional and the animals can survive for

months. *pdgfa*^{-/-} animals exhibit a loss of cells that express the PDGF receptor, while lungs isolated from *fgfr-3*^{-/-}*fgfr-4*^{-/-} and their phenotypically normal siblings exhibited comparable levels of *pdgfa* and *pdgfra* when examined with RT PCR (data not shown).

FGFR-3 and FGFR-4 function to downregulate elastin deposition

Interestingly, elastin deposition appears to be increased rather than decreased in the *fgfr-3*^{-/-}*fgfr-4*^{-/-} lungs. Previous work has shown that basic FGF can decrease elastin transcription in cultured pneumocytes either when added exogenously or when released by elastase treatment (Rich et al., 1996). It is likely that bFGF signals through FGFR-3 and FGFR-4, and that, in the absence of these receptors, elastin synthesis is increased leading to the aberrantly high deposition seen. Elastin may therefore be a de facto FGF target gene. The balance between elastin deposition and elastin degradation is extremely important for pulmonary homeostasis. Increases in the level of α_1 -antitrypsin can result in spontaneous emphysema (de Santi et al., 1995) for example, while disruption of macrophage elastase confers resistance to emphysema induced by cigarette smoke (Hautamaki et al., 1997). Aberrant elastin synthesis is unlikely to cause the observed phenotype because elastin synthesis is not elevated during alveogenesis in the double mutants (Fig. 7F), and the increased deposition is not seen in *fgfr-3*^{-/-}*fgfr-4*^{-/-} lungs until well after the abnormalities are readily observable (Fig. 7A,B and unpublished observations).

Other aspects of the double mutant lungs appear normal. Their surfactant complement is not obviously changed, although we cannot rule out a subtle alteration. The rates of cell proliferation and apoptosis appear unaffected, the latter monitored by TUNEL staining of postnatal lungs. FGFs have been shown to increase cell proliferation in a variety of biological systems. Despite this, our finding of normal proliferation in the mutant lungs suggests that it is a differentiation process, not a proliferation step that is undermined.

fgfr-3^{-/-}*fgfr-4*^{-/-} animals are viable although sickly, suggesting lung function is not completely impaired. Indeed the phenotype that we have observed exhibits similarities to neonatal bronchopulmonary dysplasia, a major health problem that results from the ventilation of premature infants (reviewed in Coalson, 1997). This syndrome can be mimicked in experimental animals by the forced ventilation of late-term embryos delivered by Cesarean section, but the *fgfr-3*^{-/-}*fgfr-4*^{-/-} animals are the first viable genetic model of this important disease.

In summary, we have shown that the *fgfr-4* gene is by itself largely dispensable for normal development, but *fgfr-3* and *fgfr-4* function cooperatively to promote the process of alveogenesis late in development and shut off synthesis of the major extracellular matrix protein, elastin. A differential display analysis of double mutant versus sibling lungs may reveal novel genes involved during the septation process, many of which may be FGF targets.

We are indebted to Philip Leder, in whose laboratory this work was initiated. We also wish to thank Cuiling Li and David Hair for technical assistance, Robin Humphries and Shanxiang Shen for

critical reading of this manuscript, and members of the Deng laboratory for suggestions and support during the course of this study.

REFERENCES

- Arman, E., Haffner-Krausz, R., Chen, Y., Heath, J. and Lonai, P. (1995). Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. *Proc. Natl Acad. Sci. USA* **95**, 5082-5087.
- Avraham, K. B., Givol, D., Avivi, A., Yayon, A., Copeland, N. G. and Jenkins, N. A. (1994). Mapping of murine fibroblast growth factor receptors refines regions of homology between mouse and human chromosomes. *Genomics* **21**, 656-8.
- Bellucci, S., Furuta, Y., Rush, M. G., Henderson, R., Winnier, G. and Hogan, B. L. (1997). Involvement of Sonic hedgehog (Shh) in mouse embryonic lung growth and morphogenesis. *Development* **124**, 53-63.
- Bellucci, S., Henderson, R., Winnier, G., Oikawa, T. and Hogan, B. L. (1996). Evidence from normal expression and targeted misexpression that bone morphogenetic protein (Bmp-4) plays a role in mouse embryonic lung morphogenesis. *Development* **122**, 1693-702.
- Bostrom, H., Willetts, K., Pekny, M., Leveen, P., Lindahl, P., Hedstrand, H., Pekna, M., Hellstrom, M., Gebre-Medhin, S., Schalling, M., Nilsson, M., Kurland, S., Tornell, J., Heath, J. K. and Betsholtz, C. (1996). PDGF-A signaling is a critical event in lung alveolar myofibroblast development and alveogenesis. *Cell* **85**, 863-73.
- Burri, P. H. (1991). Postnatal Development and Growth. In *The Lung: Scientific Foundations*, (ed. R. G. Crystal and J. B. West), pp. 677-686. New York: Raven Press, Ltd.
- Cardoso, W. V., Itoh, A., Nogawa, H., Mason, I. and Brody, J. S. (1997). FGF-1 and FGF-7 induce distinct patterns of growth and differentiation in embryonic lung epithelium. *Dev. Dynamics* **208**, 398-405.
- Chellaiah, A. T., McEwen, D. G., Werner, S., Xu, J. and Ornitz, D. M. (1994). Fibroblast growth factor receptor (FGFR) 3. Alternative splicing in immunoglobulin-like domain III creates a receptor highly specific for acidic FGF/FGF-1. *J. Biol. Chem.* **269**, 11620-7.
- Chen, F. and Capecchi, M. R. (1997). Targeted mutations in *hoxa-9* and *hoxb-9* reveal synergistic interactions. *Dev. Biol.* **181**, 186-96.
- Ciruna, B. G., Schwartz, L., Harpal, K., Yamaguchi, T. P. and Rossant, J. (1997). Chimeric analysis of fibroblast growth factor receptor-1 (*Fgfr1*) function: a role for FGFR1 in morphogenetic movement through the primitive streak. *Development* **124**, 2829-41.
- Coalson, J. J. (1997). Experimental models of bronchopulmonary dysplasia. *Biol. Neonate* **71 Suppl 1**, 35-8.
- Cohn, M. J., Izpisua-Belmonte, J. C., Abud, H., Heath, J. K. and Tickle, C. (1995). Fibroblast growth factors induce additional limb development from the flank of chick embryos. *Cell* **80**, 739-46.
- Colucci-Guyon, E., Portier, M. M., Dunia, I., Paulin, D., Pournin, S. and Babinet, C. (1994). Mice lacking vimentin develop and reproduce without an obvious phenotype. *Cell* **79**, 679-94.
- Colvin, J. S., Bohne, B. A., Harding, G. W., McEwen, D. G. and Ornitz, D. M. (1996). Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.* **12**, 390-7.
- Crossley, P. H., Martinez, S. and Martin, G. R. (1996). Midbrain development induced by FGF8 in the chick embryo. *Nature* **380**, 66-8.
- Crossley, P. H., Minowada, G., MacArthur, C. A. and Martin, G. R. (1996). Roles for FGF8 in the induction, initiation, and maintenance of chick limb development. *Cell* **84**, 127-36.
- Davis, A. P., Witte, D. P., Hsieh-Li, H. M., Potter, S. S. and Capecchi, M. R. (1995). Absence of radius and ulna in mice lacking *hoxa-11* and *hoxd-11*. *Nature* **375**, 791-5.
- de Santi, M. M., Martorana, P. A., Cavarra, E. and Lungarella, G. (1995). Pallid mice with genetic emphysema. Neutrophil elastase burden and elastin loss occur without alteration in the bronchoalveolar lavage cell population. *Lab. Invest.* **73**, 40-7.
- Deng, C., Bedford, M., Li, C., Xu, X., Yang, X., Dunmore, J. and Leder, P. (1997). Fibroblast growth factor receptor-1 (FGFR-1) is essential for normal neural tube and limb development. *Dev. Biol.* **185**, 42-54.
- Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A. and Leder, P. (1996). Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell* **84**, 911-21.
- Deng, C. X., Wynshaw-Boris, A., Shen, M. M., Daugherty, C., Ornitz, D.

- M. and Leder, P. (1994). Murine FGFR-1 is required for early postimplantation growth and axial organization. *Genes Dev.* **8**, 3045-57.
- Deterding, R. R., Jacoby, C. R. and Shannon, J. M. (1996). Acidic fibroblast growth factor and keratinocyte growth factor stimulate fetal rat pulmonary epithelial growth. *Am. J. Physiol.* **271**, L495-505.
- Feldman, B., Poueymirou, W., Papaioannou, V. E., DeChiara, T. M. and Goldfarb, M. (1995). Requirement of FGF-4 for postimplantation mouse development. *Science* **267**, 246-9.
- Floss, T., Arnold, H. H. and Braun, T. (1997). A role for FGF-6 in skeletal muscle regeneration. *Genes Dev.* **11**, 2040-51.
- Fukuda, T., Kawano, H., Ohyama, K., Li, H. P., Takeda, Y., Oohira, A. and Kawamura, K. (1997). Immunohistochemical localization of neurocan and L1 in the formation of thalamocortical pathway of developing rats. *J. Comp. Neurol.* **382**, 141-52.
- Galou, M., Colucci-Guyon, E., Ensergueix, D., Ridet, J. L., Gimenez y Ribotta, M., Privat, A., Ebinet, C. and Dupouey, P. (1996). Disrupted glial fibrillary acidic protein network in astrocytes from vimentin knockout mice. *J. Cell Biol.* **133**, 853-63.
- Glazer, L. and Shilo, B. Z. (1991). The Drosophila FGF-R homolog is expressed in the embryonic tracheal system and appears to be required for directed tracheal cell extension. *Genes Dev.* **5**, 697-705.
- Goldfarb, M. (1996). Functions of fibroblast growth factors in vertebrate development. *Cytokine Growth Factor Rev.* **7**, 311-25.
- Grindley, J. C., Belluscini, S., Perkins, D. and Hogan, B. L. (1997). Evidence for the involvement of the Gli gene family in embryonic mouse lung development. *Dev. Biol.* **188**, 337-48.
- Guo, L., Degenstein, L. and Fuchs, E. (1996). Keratinocyte growth factor is required for hair development but not for wound healing. *Genes Dev.* **10**, 165-75.
- Hautamaki, R. D., Kobayashi, D. K., Senior, R. M. and Shapiro, S. D. (1997). Requirement for macrophage elastase for cigarette smoke-induced emphysema in mice. *Science* **277**, 2002-4.
- Hebert, J. M., Rosenquist, T., Gotz, J. and Martin, G. R. (1994). FGF5 as a regulator of the hair growth cycle: evidence from targeted and spontaneous mutations. *Cell* **78**, 1017-25.
- Jaakkola, S., Salmikangas, P., Nylund, S., Partanen, J., Armstrong, E., Pyrhonen, S., Lehtovirta, P. and Nevanlinna, H. (1993). Amplification of fgfr4 gene in human breast and gynecological cancers. *Int. J. Cancer* **54**, 378-82.
- Johnson, D. E. and Williams, L. T. (1993). Structural and functional diversity in the FGF receptor multigene family. *Adv. Cancer Res.* **60**, 1-41.
- Kaartinen, V., Voncken, J. W., Shuler, C., Warburton, D., Bu, D., Heisterkamp, N. and Groffen, J. (1995). Abnormal lung development and cleft palate in mice lacking TGF-beta 3 indicates defects of epithelial-mesenchymal interaction. *Nat. Genet.* **11**, 415-21.
- Kimura, S., Hara, Y., Pineau, T., Fernandez-Salguero, P., Fox, C. H., Ward, J. M. and Gonzalez, F. J. (1996). The T/ebp null mouse: thyroid-specific enhancer-binding protein is essential for the organogenesis of the thyroid, lung, ventral forebrain, and pituitary. *Genes Dev.* **10**, 60-9.
- Lee, T., Hacohen, N., Krasnow, M. and Montell, D. J. (1996). Regulated Breathless receptor tyrosine kinase activity required to pattern cell migration and branching in the Drosophila tracheal system. *Genes Dev.* **10**, 2912-21.
- Mansour, S. L., Goddard, J. M. and Capocchi, M. R. (1993). Mice homozygous for a targeted disruption of the proto-oncogene int-2 have developmental defects in the tail and inner ear. *Development* **117**, 13-28.
- Mariani, T. J., Sandefur, S. and Pierce, R. A. (1997). Elastin in lung development. *Exp. Lung Res.* **23**, 131-45.
- Masters, J. R. (1976). Epithelial-mesenchymal interaction during lung development: the effect of mesenchymal mass. *Dev. Biol.* **51**, 98-108.
- McWhirter, J. R., Goulding, M., Weiner, J. A., Chun, J. and Murre, C. (1997). A novel fibroblast growth factor gene expressed in the developing nervous system is a downstream target of the chimeric homeodomain oncoprotein E2A-Pbx1. *Development* **124**, 3221-32.
- Moens, C. B., Auerbach, A. B., Conlon, R. A., Joyner, A. L. and Rossant, J. (1992). A targeted mutation reveals a role for N-myc in branching morphogenesis in the embryonic mouse lung. *Genes Dev.* **6**, 691-704.
- Muenke, M. and Schell, U. (1995). Fibroblast-growth-factor receptor mutations in human skeletal disorders. *Trends Genet.* **11**, 308-13.
- Niswander, L., Jeffrey, S., Martin, G. R. and Tickle, C. (1994). A positive feedback loop coordinates growth and patterning in the vertebrate limb [see comments]. *Nature* **371**, 609-12.
- Ohuchi, H., Nakagawa, T., Yamamoto, A., Araga, A., Ohata, T., Ishimaru, Y., Yoshioka, H., Kuwana, T., Nohno, T., Yamasaki, M., Itoh, N. and Noji, S. (1997). The mesenchymal factor, FGF10, initiates and maintains the outgrowth of the chick limb bud through interaction with FGF8, an apical ectodermal factor. *Development* **124**, 2235-44.
- Ozawa, K., Uruno, T., Miyakawa, K., Seo, M. and Imamura, T. (1996). Expression of the fibroblast growth factor family and their receptor family genes during mouse brain development. *Brain Res. Mol. Brain Res.* **41**, 279-88.
- Peters, K., Werner, S., Liao, X., Wert, S., Whitsett, J. and Williams, L. (1994). Targeted expression of a dominant negative FGF receptor blocks branching morphogenesis and epithelial differentiation of the mouse lung. *EMBO J.* **13**, 3296-301.
- Rich, C. B., Nugent, M. A., Stone, P. and Foster, J. A. (1996). Elastase release of basic fibroblast growth factor in pulmonary fibroblast cultures results in down-regulation of elastin gene transcription. A role for basic fibroblast growth factor in regulating lung repair. *J. Biol. Chem.* **271**, 23043-8.
- Riddle, R. D., Johnson, R. L., Laufer, E. and Tabin, C. (1993). Sonic-hedgehog mediates the polarizing activity of the zpa. *Cell* **75**, 1401-1416.
- Sibilia, M. and Wagner, E. F. (1995). Strain-dependent epithelial defects in mice lacking the EGF receptor [published erratum appears in Science 1995 Aug 18;269(5226):909]. *Science* **269**, 234-8.
- Simonet, W. S., DeRose, M. L., Bucay, N., Nguyen, H. Q., Wert, S. E., Zhou, L., Ulich, T. R., Thomason, A., Danilenko, D. M. and Whitsett, J. A. (1995). Pulmonary malformation in transgenic mice expressing human keratinocyte growth factor in the lung. *Proc. Natl Acad. Sci. USA* **92**, 12461-5.
- Smallwood, P. M., Munoz-Sanjuan, I., Tong, P., Macke, J. P., Hendry, S. H., Gilbert, D. J., Copeland, N. G., Jenkins, N. A. and Nathans, J. (1996). Fibroblast growth factor (FGF) homologous factors: new members of the FGF family implicated in nervous system development. *Proc. Natl Acad. Sci. USA* **93**, 9850-7.
- Stark, K. L., McMahon, J. A. and McMahon, A. P. (1991). FGFR-4, a new member of the fibroblast growth factor receptor family, expressed in the definitive endoderm and skeletal muscle lineages of the mouse. *Development* **113**, 641-51.
- Sutherland, D., Samakovlis, C. and Krasnow, M. A. (1996). branchless encodes a Drosophila FGF homolog that controls tracheal cell migration and the pattern of branching. *Cell* **87**, 1091-101.
- Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T. and Mulligan, R. C. (1991). Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. *Cell* **65**, 1153-63.
- Vainikka, S., Joukov, V., Klint, P. and Alitalo, K. (1996). Association of a 85-kDa serine kinase with activated fibroblast growth factor receptor-4. *J. Biol. Chem.* **271**, 1270-3.
- Vainikka, S., Partanen, J., Bellosta, P., Coulier, F., Birnbaum, D., Basilico, C., Jaye, M. and Alitalo, K. (1992). Fibroblast growth factor receptor-4 shows novel features in genomic structure, ligand binding and signal transduction [published erratum appears in EMBO J 1993 Feb;12(2):810]. *EMBO J.* **11**, 4273-80.
- Verdier, A. S., Mattei, M. G., Lovec, H., Hartung, H., Goldfarb, M., Birnbaum, D. and Coulier, F. (1997). Chromosomal mapping of two novel human FGF genes, FGF11 and FGF12. *Genomics* **40**, 151-4.
- Vogel, A., Rodriguez, C. and Izpisua-Belmonte, J. C. (1996). Involvement of FGF-8 in initiation, outgrowth and patterning of the vertebrate limb. *Development* **122**, 1737-50.
- Xu, X., Weinstein, M., Li, C., Naske, M., Cohen, R. I., Ornitz, D., Leder, P. and Deng, C. (1998). Fibroblast growth factor receptor 2 (FGFR2) is required for placental and limb bud induction. *Development* **125**, 753-765.
- Yamaguchi, T. P., Harpal, K., Henkemeyer, M. and Rossant, J. (1994). fgfr-1 is required for embryonic growth and mesodermal patterning during mouse gastrulation. *Genes Dev.* **8**, 3032-44.
- Yamasaki, M., Miyake, A., Tagashira, S. and Itoh, N. (1996). Structure and expression of the rat mRNA encoding a novel member of the fibroblast growth factor family. *J. Biol. Chem.* **271**, 15918-21.