An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis

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SUMMARY

The fibroblast growth factor receptor 2 gene is differentially spliced to encode two transmembrane tyrosine kinase receptor proteins that have different ligand-binding specificities and exclusive tissue distributions. We have used Cre-mediated excision to generate mice lacking the IIIb form of fibroblast growth factor receptor 2 whilst retaining expression of the IIIc form. Fibroblast growth factor receptor 2(IIIb) null mice are viable until birth, but have severe defects of the limbs, lung and anterior pituitary gland. The development of these structures appears to initiate, but then fails with the tissues undergoing extensive apoptosis. There are also developmental abnormalities of the salivary glands, inner ear, teeth and skin, as well as minor defects in skull formation. Our findings point to a key role for fibroblast growth factor receptor 2(IIIb) in mesenchymal-epithelial signalling during early organogenesis.

Key words: Fibroblast growth factor receptor 2, IIIb isoform, Limb development, Skin, Pituitary gland, Lung

INTRODUCTION

Fibroblast growth factors (Fgfs) are a large family of intercellular signalling molecules, whose activities are mediated through a family of tyrosine kinase transmembrane receptors (reviewed in Johnson and Williams, 1993; McKeehan et al., 1998). Binding of FGFs to their receptors is facilitated by heparan sulphate-containing proteoglycans and culminates in activation of the receptor tyrosine kinase and signal transduction. Fgfs have been implicated in the development of a diverse range of animal species from Caenorhabditis elegans to mammals (for examples, see Amaya et al., 1991; DeVore et al., 1995; Reichman-Fried and Shilo, 1995; Yamaguchi and Rossant, 1995). Moreover, genetic linkage analysis has implicated three members of the FGF receptor (Fgfr) gene family as the underlying cause of several skeletal dysplasias and autosomal dominant craniosynostosis syndromes (reviewed in DeMoerlooze and Dickson, 1997).

Four FGF receptor genes have been identified in mammals (Fgfr1 to Fgfr4), each comprising an extracellular domain composed of two or three immunoglobulin-like (Ig) loops, a transmembrane segment and an intracellular tyrosine kinase (reviewed in Johnson and Williams, 1993; McKeehan et al., 1998). Ligand-binding specificity of the receptor is mediated by the second and third Ig-loop. For Fgfr1-Fgfr3, the third Ig loop is encoded by two exons, an invariant exon termed IIIa and one of two exons, termed IIIb and IIIc, respectively, to which the IIIa exon is spliced. This generates two receptor isoforms with quite different ligand-binding specificities (see Ornitz et al., 1996). Fgfr2 containing the IIIb exon is found mainly in epithelia, and is activated by four known ligands (Fgf1, Fgf3, Fgf7 and Fgf10), which are synthesized predominantly in the tissue mesenchyme. In contrast, Fgfr2(IIIc) is located primarily in the mesenchyme, and apart from Fgf1, which binds to all known receptors, is activated by a different set of Fgf ligands (Bellusci et al., 1997; Finch et al., 1995; Mason et al., 1994; Ornitz et al., 1996; Orr-Urtreger et al., 1993; Peters et al., 1992; Yamasaki et al., 1996). Hence, Fgfr2(IIIb) and Fgfr2(IIIc) are expressed in mutually exclusive cell lineages, using a positively regulated splicing mechanism that involves intron sequences adjacent to the isoform-specific exons (Carstens et al., 1997, 1998; Del Gatto et al., 1997; Gilbert et al., 1993).

The importance of FGF receptors in mouse development has been established by gene targeting studies, which show that mice deficient for Fgfr1 die during gastrulation with both impaired growth and cell migration through the primitive streak (Ciruna et al., 1997; Deng et al., 1994, 1997; Yamaguchi et al., 1994). A null mutation encompassing both isoforms of Fgfr2 results in peri-implantation lethality at E4.5-E5.5 (Arman et al., 1998), while embryos with a homozygous hypomorphic allele die around E10.5 with no limb buds and a defective placenta (Xu et al., 1998). A targeted deletion of Fgfr3 results in skeletal dysplasia of the long bones and an
inner ear defect (Colvin et al., 1996; Deng et al., 1996). Interestingly, Fgfr4-deficient mice show no apparent phenotype, but the Fgfr3/Fgfr4 double null demonstrates a late lung defect not found in the single receptor-deficient mice (Weinstein et al., 1998).

Expression of the isoform-specific dominant negative Fgfr2(IIIb) implicates this receptor in the development of several organs including the limbs, lungs, skin, kidney and several glandular tissues (Celi et al., 1998; Jackson et al., 1997; Peters et al., 1994; Robinson et al., 1995; Werner et al., 1993). To understand the role of Fgfr2(IIIb) in mouse development, we have specifically abrogated its expression in the mouse germ line while leaving intact a functional Fgfr2(IIIc). Unlike the complete Fgfr2 null mutation which is peri-implantation lethal, mice lacking the IIIb-specific isoform of Fgfr2 are viable until birth. However, these mutant mice display a wide range of phenotypic abnormalities implicating this receptor isoform in the induction and specification of several cell lineages in multiple organs during mouse development.

MATERIALS AND METHODS

Gene targeting

Recombinant lambda bacteriophage encompassing the alternatively spliced region of Fgfr2 were isolated from a 129SvJ-mouse genomic library (kind gift of A.-M. Frischauf). To construct the targeting vector a 96 bp loxP recognition sequence was inserted into the intron between exons IIIb and IIIC at an XbaI site. The plasmid pH1-1 (Gu et al., 1993), which encodes a loxP site 5′ to HSV-neo and HSV-TK genes, was modified by the addition of a loxP sequence at the 3′ end of HSV-TK. This neo/TK cassette flanked by lox-P sites was inserted at the BamHI site in the intron between exons IIIa and IIIb. The final construct was approximately 8.5 kb in length, starting at an introduced XhoI site in the intron upstream of exon IIIa and terminating at an XbaI site in the intron 3′ of exon IIIC (Fig. 1). The plasmid DNA was electroporated into G1K12 ES cells as previously described (Famili et al., 1995) and HindIII-digested DNA from neo-resistant clones screened by Southern hybridization with an internal probe. Candidate homologous recombinant clones were re-screened using PCR with primers that would amplify the 5′ and 3′ junction fragments of correctly targeted DNA. To generate ES cells with a conditional or non-conditional Fgfr2(IIIb) allele, they were transiently transfected with pMC-Cre (Gu et al., 1993) plasmid, which expresses the Cre recombinase from the CMV promoter, and resorted for the loss of the plasmid cassette in the presence of gancyclovir. Several ES cell clones were isolated from two independent primary ES clones that were either non-conditional or conditional for the IIIB exon of Fgfr2 (see Fig. 1). ES cell clones were used to generate chimeras. We obtained germine transmission of two conditional ES cell clones. Mice with the non-conditional allele were obtained by injecting fertilized C57Bl/6 × Fgfr2(IIIb)−/−cond eggs with pCAGGS-Cre (Araki et al., 1995).

Genotyping

Genomic DNA was isolated from tail clips (Laird et al., 1991) and genotype determined using two pairs of primers P1/P2 and P3/P4 as shown in Fig. 1. P1 is in the IIIB exon and therefore absent in the excised allele and P4 is in the loxP site and absent in WT. The PCR protocol was 94°C 2 minutes, then 30 cycles of 94°C 30 seconds, 55°C 1 minute, 72°C 30 seconds, followed by 72°C 10 minutes, using a Techne Genius (Techne). Products were run out on a 1% agarose gel. Primer sequences available on request.

RT-PCR analysis

Total RNA was prepared from snap-frozen adult and fetal tissues using TRIzol Reagent (Gibco BRL) according to the manufacturer’s protocol and 1 μg of each RNA was used with Ready-To-Go RT-PCR Beads (Pharmacia Biotech) according to the manufacturer’s recommended one-step protocol. The following primers were used: 5′ IIIa, AAGGTTTACAGGTATGCCCA; 3′ TM, ACCACATGCAGCCATTAA. PCR conditions: 94°C/1 minute, 47°C/1 minute, 72°C/1 minute, for 35 cycles then 72°C/10 minutes. PCR products were analyzed on a 2% agarose gel. Total reactions and isolated DNA bands were restriction digested with AvaI or EcoRV to discriminate between products containing IIIb and IIIc exons, respectively.

Histology and immunohistochemistry

Tissue was fixed overnight in neutral buffered formalin, dehydrated through ethanol and embedded in paraffin wax, and 5 μm sections were cut for staining in Haematoxylin and Eosin. For immunohistochemistry, 8 μm sections were cut and, where necessary, antigens were unmasked by prior boiling for 10 minutes in 0.01 M tri-sodium citrate pH 6. A standard indirect immunoperoxidase protocol was employed. The following primary antibodies were used: ERLI-3 rabbit anti-mouse involucrin (a kind gift from F. Watt), rabbit anti-major cytokeratins (BAbCO, USA), anti-mouse cytokeratins 1, 5, 6, 10, 14 and loricrin (BAbCO, USA) at the recommended concentrations for 1 hour at room temperature. Secondary antibody was biotinylated swine anti-rabbit IgG (DAKO, UK) followed by streptavidin-peroxidase (DAKO, UK) and 3′-diaminobenzidine (Sigma, UK) visualization. Proliferating cells were detected using a rabbit antiserum to NCL-Ki67p (Novocastra, UK) as above. Apoptotic cells were detected by labeling free 3′OH ends of fragmented DNA with terminal transferase and tagged NTPs (TUNEL) using a commercial kit (ApopTag® Plus In Situ Apoptosis Detection Kit, Oncor, USA). Skeletal staining was carried out as previously described (McLeod, 1980).

In situ hybridization

A mouse FGFR2 IIIc probe was kindly provided by P. Kettunen (Kettunen et al., 1998). A mouse cDNA corresponding to the entire IIIB exon of FGFR2 IIIb (nucleotides 1267-1417; (Miki et al., 1992)) was generated by PCR and cloned into pBluescript IISK+. The Fgfr2-TK probe was a PCR-generated fragment of the cytoplasmic domain encompassing nucleotides 1726-2764 in pBluescript IISK+. Plasmids were linearized and sense and antisense single-stranded RNA probes generated with the appropriate RNA polymerase. In situ hybridization was performed on dewaxed tissue sections essentially as described previously (Kettunen et al., 1998).

RESULTS

Targeted disruption of the IIIB isoform of FGF receptor 2

A non-conditional isoform-specific mutation of Fgfr2(IIId) was obtained by removing exon IIId from the mouse germline. This was achieved using Cre recombination to excise exon IIId from the genome of targeted ES cells in a strategy outlined in Fig. 1 (Gu et al., 1993). To determine the consequences of deleting exon IIId on transcript expression, we used RT-PCR to investigate the presence of possible RNA species (Fig. 2). A pair of primers was designed to detect the possible alternative exon splicing events; namely exons IIId to IIIc, IIId to IIIBC and IIId to TM. As the size of the products generated from transcripts for wild-type IIId and IIIdc receptor isoforms is very similar (340 verses 345 nts), we used restriction endonuclease digestion at sites unique to each product to help distinguish them. The results show that wild-type mice exhibited the...
splicing patterns for the (IIIb) and (IIIc) isoforms of Fgfr2 as expected. By contrast, the Fgfr2(IIIb)-/- mice showed the splicing pattern for the Fgfr2(IIIc), and a novel splice generated by splicing exon IIIa directly to exon TM (Fig. 2). Heterozygous mutant mice showed all three splicing alternatives. The IIIa to TM exon splice causes translation to terminate two codons into TM. The resulting product lacks half of Ig-loop three (part of the ligand-binding domain), the transmembrane element as well as the kinase (Fig. 2). Therefore, it is highly unlikely that this severely truncated protein could have any significant receptor activity. As a further check on receptor splicing, we have used in situ hybridisation on skin sections to confirm the differential expression of Fgfr2(IIIb), Fgfr2(IIIc) and the aberrant receptor RNA (described below).

Skeletal and craniofacial abnormalities
Heterozygous mice were indistinguishable from their wild-
type littermates in all respects and have remained healthy for more than a year. Interbreeding of heterozygous mutant mice resulted in approximately 25% homozygous mutant embryos, indicating no significant lethality in utero due to the loss of the Fgfr2(IIIb) receptor isoform. The homozygous mutant fetuses survived to term but died at birth due to failure of lung formation (see below). They were smaller than their littermates, and had no forelimbs or hindlimbs, an abnormally curled tail and open eyes due to an apparent absence of eyelids (Fig. 3A,B). To investigate the skeletal changes, fetuses were stained with Alizarin red and Alcian blue to show bone and cartilage structures, respectively (McLeod, 1980); Fig. 3C,D). The skeletal stain confirmed the absence of limb rudiments in Fgfr2(IIIb)−/− mice and showed that the scapula, particularly the blade and coracoid process, was greatly reduced in size and the acromion failed to form (Fig. 3E). The clavicle, which normally joins to the acromion, was fused to the coracoid process. The Fgfr2(IIIb)−/− mice also showed a severely malformed pelvic girdle, with only a rudimentary ileum and ischium, and the absence of discernible pubis and ischial rami (Fig. 3E). An examination of the tail at E16.5 showed malformed and fused vertebrae that would account for the curled tail phenotype (Fig. 3F,G).

The heads of homozygous mutant fetuses appeared more domed than their heterozygous or wild-type counterparts (Fig. 3A,B), and skeletal staining revealed an apparent premature fusion of the suture between the parietal and squamous temporal bones (Fig. 4). In addition, the skulls of Fgfr2(IIIb)−/− fetuses showed otic capsules of greatly reduced size and cleft palate.

**Failure of limb bud initiation**

To investigate the stage at which limb formation fails, embryos from earlier stages of development were examined. E10.5 Fgfr2(IIIb)−/− embryos were the most revealing, since they showed the beginning of limb bud initiation but no thickening of the epithelium characteristic of an apical endodermal ridge (Fig. 5A,B). Moreover, TUNEL staining of adjacent sections showed extensive apoptosis in the presumptive limb bud mesoderm, as well as some apoptotic cells in the overlying epithelium (Fig. 5C,D). By contrast, the control wild-type limb buds showed a well-demarcated AER and virtually no apoptotic cells.

**Non-skeletal defects of the head and neck**

Dissection and serial sectioning of the ear from homozygous mutants demonstrated that semicircular canal formation was grossly abnormal and the whole inner ear was generally cystic in appearance (data not shown). The malformation of the inner ear can in part be explained by an earlier failure, in some mice, of endolymphatic duct formation as shown in sections of E10.5 embryos (Fig. 6).

Sagittal sections of the head region from E12.5-16.5 embryos revealed several other abnormalities. The teeth of Fgfr2(IIIb)−/− mutant mice did not develop normally and failed to progress beyond the bud stage, which normally occurs at E13.5 (Fig. 7A,B) (Thesleff and Sharpe, 1997). Embryos from E12.5 to E16.5 were examined for the development of salivary glands. As shown in Fig. 7C,D, E14.5 Fgfr2(IIIb)−/− embryos showed clear deficiencies in salivary gland formation. Transverse and sagittal sectioning of E12.5 and E14.5 embryos demonstrated the absence of an anterior pituitary and its precursor, Rathke’s pouch (Fig. 8A,B). However, the infundibular recess and its derivative, the neural component of...
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the pituitary (pars nervosa), was present and appeared morphologically normal. At E9.5, Rathke’s pouch starts to form from the oral cavity ectoderm and, a half day later, the infundibular recess derives from the neural epithelium (Kaufman, 1992). Coronal sections through E10.5 embryos show these processes in both wild-type and homozygous mutant embryos (Fig. 8C,D). However, the appearance of Rathke’s pouch in the \( Fgfr2(IIIb)^{-/-} \) embryos appears poorly formed with fewer cell layers forming the walls of the diverticulum. Furthermore, TUNEL staining shows widespread apoptosis in the pouch epithelium of \( Fgfr2(IIIb)^{-/-} \) embryos (Fig. 8C-F). Wild-type embryos showed only a few apoptotic cells at the base of the diverticulum, consistent with the formation of Rathke’s pouch from the oral epithelium.

**Visceral abnormalities**

Dissection of the thoracic cavity of \( Fgfr2(IIIb) \)-deficient E18.5 fetuses showed a clear absence of lungs. However, defective lung development was already apparent much earlier at E10.5. The tracheal bifurcation which generates the bronchi was evident in heterozygous and wild-type embryos, but was not observed in \( Fgfr2(IIIb)^{-/-} \) embryos (Fig. 9A,B). However, sagittal sections of E12.5 embryos showed a finger of mesenchyme surrounded by a single layer of epithelium but, by E14.5, the mesenchyme is full of picnotic nuclei, a sign of extensive apoptosis (Fig. 9D,F). By contrast, the normal mouse lung at E14.5 showed considerable bronchi with branching and bronchioles that were beginning to differentiate (Fig. 9C,E). The stomach also appeared relatively smaller in mutant fetuses and had some differences in tissue architecture compared to heterozygous mice (data not shown).

**Skin development**

The skin of the \( Fgfr2(IIIb)^{-/-} \) mice was lighter in colour and more translucent compared to the normal pink wrinkled appearance of their heterozygous and wild-type littermates (Fig. 3A,B). Transverse sections through the dorsal skin of the mutant mice demonstrated a marked reduction in thickness that accounted, at least in part, for its appearance (Fig. 11A,E). To analyse the phenotype in more detail, we used an immunohistochemical approach to compare a number of differentiation markers. Examples of the staining patterns for cytokeratins 1 and 5, and loricrin are shown in Fig. 10. In essence, all the normal cell lineages found in heterozygous and wild-type skin were detected in their correct relative position within the mutant skin, indicating that epidermal differentiation was grossly similar. However, the different stratified layers of the epidermis and the dermis were significantly thinner and appeared to be less well

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**Fig. 4.** Skull abnormalities at E19.5. (A,C,E) Heterozygous mouse; (B,D,F) \( Fgfr2(IIIb)^{-/-} \) mouse. (A,B) Ventral view of the skulls; asterisk, an otic capsule. (C,D) Lateral views of A and B, respectively. An incisor is indicated by the arrow in C. Dashed boxes indicate the region of premature suture formation presented at higher magnification in E and F; p, parietal bone; sq, the squamous temporal bone.

**Fig. 5.** Limb bud defects. (A,C) Sections of wild-type forelimb buds at E10.5 stained with H&E and by TUNEL, respectively. (B,D) Equivalent sections taken and stained from a null littermate. Asterisk, forelimb bud; arrowhead in A, AER; arrowheads in D, brown immunoperoxidase staining of apoptotic cells in the ectoderm. Note too, the widespread immunoperoxidase staining of the mesoderm. The position of the AER is indicated in C. This structure is missing in null mice. Bars, 100 µm.
organised, particularly those of the basal keratinocyte layer between the hair follicles. More significantly, in the Fgfr2(IIIb)−/− fetuses, there was no discernible panniculus carnosus muscle layer that normally lies beneath the dermis (Fig. 11A,E). To investigate cell proliferation rates, an antibody to Ki-67, which stains cells in the division cycle, was used and a decrease in the number of cycling cells was observed in the basal layer of the homozygous mutant mice. However, staining of the outer hair follicle sheath appeared similar for all genotypes. Apoptotic cells were detected by TUNEL staining as described. Few cells appeared to stain in this assay and there was no noticeable difference between mutant and control skin samples (data not shown).

Fgfr2 expression in the skin

As mouse skin is well characterised in terms of its cellular architecture and expression of Fgfr2, it was used to further investigate the splicing pattern of the receptor in normal and mutant fetuses (Orr-Urtreger et al., 1993; Peters et al., 1992).

In situ hybridisation was performed with probes to the IIIb and IIIc exons and the TK domain of the Fgfr2 receptor (Fig. 11). The basal keratinocyte layer and the hair follicle sheaths of the heterozygous and wild-type skin were strongly positive with probes against the TK and IIIb domains of Fgfr2, while a probe from the IIIc exon gave a weaker hybridization signal in the dermis and panniculus carnosus. The mutant embryos did not express the IIIb exon as expected. Nevertheless, the TK probe gave a similar pattern of expression on mutant and wild-type skin sections, indicating that despite the removal of the IIIb exon in mutant embryos, similar levels of Fgfr2 RNA were present. This finding is consistent with the IIIa to TM switch occurring in the absence of the IIIb exon (Fig. 2), and agrees with previous studies demonstrating that splicing of the receptor isoforms is mutually exclusive and positively regulated (Carstens et al., 1997, 1998; Del Gatto et al., 1997; Gilbert et al., 1993).

DISCUSSION

We have used the Cre-loxP recombination system to generate mice that are specifically deficient for the IIIb isoform of Fgfr2, while leaving the IIIc isoform intact (Gu et al., 1993). The results of RT/PCR analysis show that, in the absence of exon IIIb, a novel mRNA is created that splices directly from exon IIIa to TM. This behaviour is in agreement with previous studies that have demonstrated a tight regulation of alternative splicing in different cell types (Carstens et al., 1997, 1998; Del Gatto et al., 1997; Gilbert et al., 1993). The predicted truncated receptor would comprise a defective ligand-binding domain, and no transmembrane or tyrosine kinase domain. Therefore, it could not function as a signalling receptor and its defective ligand-binding domain makes it unlikely that it could serve as a dominant negative receptor. In situ hybridisation results on skin sections using IIIb- and IIIc-specific probes are consistent with the RT/PCR results and show the loss of IIIb-specific signals in the homozygous mutant mice. Moreover, by using a TK-specific probe abundant Fgfr2 RNA can be detected in the cell types that normally express Fgfr2(IIIb), indicating replacement by the IIIa/TM spliced RNA. It is also worth noting that heterozygous mice of greater than one year of age have shown no abnormalities, suggesting no gain of function results from removing exon IIIb.

Mice lacking a functional Fgfr2 receptor gene die around implantation, while we show here that mice specifically deficient for the IIIb isoform of Fgfr2 survive to birth, although they
cannot survive outside the uterus as they have no lungs (Fig. 9). At E10.5, wild-type mice show the presence of bilateral primary bronchi surrounded by mesenchyme, whereas Fgfr2(IIIb)-/- mice develop a tracheal bud consisting primarily of mesenchyme but without apparent bronchi. Several lines of investigation have implicated Fgfr2(IIIb) and its major ligands in lung morphogenesis. Expression of a dominant negative form of Fgfr2(IIIb) in the bronchial epithelium resulted in an inhibition of branching morphogenesis after primary bronchial bifurcation (Peters et al., 1994). Moreover, a similar lung phenotype to that described here was found in Fgf10-/- mice (Min et al., 1998; Sekine et al., 1999). This is consistent with Fgf10 acting as a major ligand for Fgfr2(IIIb) and its expression in the lung mesenchyme prior to the initiation of lung bud branching (Bellusci et al., 1997; Igarashi et al., 1998; Xu et al., 1998). Interestingly, the lung mesenchyme also expresses Fgf7, which exclusively activates Fgfr2(IIIb), but this ligand does not substitute for Fgf10 as an inducer of lung branching morphogenesis (Bellusci et al., 1997; Mason et al., 1994; Ornitz et al., 1996). Furthermore, mice lacking Fgf7 appear to have normal lungs (Guo et al., 1996). This would suggest either a subtle difference in spatial expression pattern, or signalling potential, between Fgf7 and Fgf10 (Bellusci et al., 1997; Mason et al., 1994). Some evidence for the latter comes from embryonic lung explant studies which show different responses to these ligands (Bellusci et al., 1997; Cardoso et al., 1997).

The absence of the anterior pituitary in Fgfr2(IIIb)-deficient mice is of particular interest, since its induction has been associated with Fgf8 (Ericson et al., 1998; Treier et al., 1998). Although Fgf8 is not a ligand for Fgfr2(IIIb), signalling through this receptor is associated with the subsequent activation of Fgf8 during limb bud initiation. Rathke’s pouch is first seen at E9.5 as a pocket arising from the roof of the oral cavity ectoderm and, approximately 12 hours later, the infundibular recess starts to form as a pocket from, and is continuous with, the neural ectoderm of the diencephalon (Kaufman, 1992). Fgf8 is strongly expressed throughout the infundibulum from as early as E10 and continues to be expressed as the anterior pituitary undergoes lineage specification, expansion and terminal differentiation to corticotropes, thyrotropes, gonadotropes, somatotropes and lactotropes. More pertinent for Fgfr2(IIIb) involvement, Fgf10 mRNA is strongly expressed in the rat infundibulum at a stage approximately equivalent to E12 in the mouse (Yamasaki et al., 1996), and studies are currently underway to establish whether Fgf10 expression occurs earlier when induction is thought to take place. However, it is clear that, in the homozygous mutant mice, there is an initial inductive phase, which starts to produce a diverticulum from the oral epithelium but, by E10.5, the structure is undergoing apoptotic disintegration (Fig. 8). Several organs including the brain,
heart, liver and kidney showed no discernible abnormalities in the null embryos up to E18.5. Mice null for Fgf7 have kidneys with a decreased volume by E16, and a reduced number of nephrons was observed in adult animals (Qiao et al., 1999). We were unable to examine postnatal tissue development as the Fgfr2(IIIb)−/− mice die at birth, and the fetuses are too abnormal to make a meaningful assessment of kidney size to body weight.

The homozygous mutant mice showed a number of skeletal abnormalities. The most obvious was the lack of forelimbs, hindlimbs and defective scapulae and pelvic girdle (Fig. 3). Two Fgfs have been implicated in the initiation and formation of the early limb; Fgf10, which is expressed in the lateral plate mesoderm prior to overt limb bud initiation (Ohuchi et al., 1997), and Fgf8, which subsequently appears in the apical ectodermal ridge (AER) and stimulates limb bud outgrowth (Crossley et al., 1996; Mahmood et al., 1995; Ohuchi et al., 1997; Vogel et al., 1996). Fgfr2(IIIb) is expressed in the ectoderm of the presumptive limb bud, which gives rise to the AER (Orr-Urtreger et al., 1993; Xu et al., 1998). The results reported here support these previous findings and show that presumptive limb buds appear but fail to form a discernible AER. Moreover, by E10.5, there is already a marked amount of apoptosis in the mesoderm and overlying epithelium. Evidence for the involvement of Fgfr2 in limb initiation was suggested by mice homozygous for a hypomorphic mutant allele of this receptor, which showed no limb bud initiation, and limb defects of variable severity were found in mice expressing a soluble dominant negative form of Fgfr2(IIIb) (Celli et al., 1998; Xu et al., 1998). Finally, mice deficient for Fgf10 were also shown to lack limbs (Min et al., 1998; Sekine et al., 1999). Taken together, there is compelling evidence that Fgf10 in the lateral plate mesoderm initiates limb development by signalling through Fgfr2(IIIb) to induce formation of the AER and subsequent Fgf8 expression.

The skulls of Fgfr2(IIIb)-deficient mice showed a thinner mandible, the absence of visible incisors and a cleft palate, phenotypic defects similar to those found for mice deficient for the homeobox gene Msx1 (Satokata and Maas, 1994). This homeobox transcription factor is associated with mesenchymal-epithelial interactions, particularly for bone and teeth, and

![Fig. 10. Immunohistochemical staining for skin differentiation and proliferation markers. Transverse sections of dorsal skin from heterozygous (A-D) and Fgfr2(IIIb)−/+ fetuses (E-H) at E18.5 stained with antibodies to cytokeratin 1 (A,E), cytokeratin 5 (B,F), loricrin (C,G) and Ki67 (D,H). Bar, 100 μm.](image)

![Fig. 11. In situ hybridization of dorsal skin to examine the expression of Fgfr2 isoforms. Skin sections from Fgfr2(IIIb)−/+ fetuses (A-D) and Fgfr2(IIIb)−/− fetuses (E-H) were taken at E18.5. (A,E) Sections (same as B,F) stained with Giemsa in bright-field illumination to reveal the cellular structure of the skin. pc, panniculus carnosus layer in the heterozygous skin absent in Fgfr2(IIIb)−/− mice. For the in situ hybridisation analysis, the following antisense probes were used: (A,B,E,F) Fgfr2-TK, (C,G) Fgfr2(IIIb), (D,H) Fgfr2(IIIc). Bar, 100 μm.](image)
including the limb, AER, facial primordia and dental follicle mesenchyme. This suggests that Fgf signalling might be closely linked, upstream or downstream to Msx gene expression. Abnormal tooth development could also represent a further link between Msx1 and Fgf signalling.

To date, many of the Fgrf2 mutations that underlie the human craniosynostosis syndromes are found specifically in the IIIc exon, but not the IIb exon, of this receptor (DeMoorloze and Dickson, 1997). This would suggest that Fgrf2(IIb) signalling is not essential for suture formation. It is therefore interesting that we found premature suture ossification associated with the Fgrf2(IIb) signaling. However, it is now known that Fgrf2(IIb) is expressed in differentiating osteoblasts at the osteogenic fronts and therefore may have a role in normal suture formation (David Rice, personal communication).

Two other aspects of the phenotype correlate with the defects observed for Fgrf3 null mice (Mansour et al., 1993). The fusion of the caudal vertebrae giving rise to a curved tail (Fig. 3) and the absence, in some mice, of an endolympathic duct (Fig. 6), which must contribute to the later inner ear malformations. These abnormalities are consistent with previous findings that show Fgf3 signals through the IIb isoform of Fgrf2 and it is expressed in the otic vesicle at E10.5 (Mathieu et al., 1995; Wilkinson et al., 1989).

The skin of homozygous mutant mice was clearly abnormal since the thickness of the dermis and epidermis were reduced compared with control mice (Figs 10, 11). In addition, the presumptive eyelids were absent in Fgrf2(IIb) null fetuses and may correlate with the normal expression of this receptor in the underlying corneal epithelium (Orr-Urtreger et al., 1993). However, an analysis of differentiation markers for skin revealed a similar pattern of expression for cytokeratins 1, 5, 6, 10, 14 as well as loricrin and involucrin (Fig. 10 and data not shown), suggesting that there is an overall effect on proliferation but little effect on differentiation. At this stage of development, we could not exclude some functional defect of the hair follicle. This result is somewhat different from two studies which have used dominant negative Fgrf2(IIb) receptors (Celli et al., 1998; Werner et al., 1994). Targeting of a membrane-located dominant negative Fgrf2(IIb) to basal keratinocytes resulted in epidermal atrophy and abnormalities in the hair follicles and a hyperthickening of the dermis. However, expression of a soluble dominant negative Fgrf2(IIb) in the skin caused a reduction of the dermis and epidermis, similar to the Fgrf2+/− mice, but, for some mice, there were no hair follicles and the inappropriate expression of cytokeratin 6, which is usually silent in normal skin. These last two features were not part of the Fgrf2(IIb) signaling phenotype. These differences probably arise from the fact that dominant negative receptors homodimerize and heterodimerize with other Fgf receptors that bind to the same ligand giving rise to a less specific inhibition of Fgf signalling. Thus, depending on context, a dominant negative receptor could inhibit signalling through several receptors and therefore give a more severe phenotype than its equivalent germ line deletion.

Many of the observed abnormalities found in Fgrf2(IIb)−/− mice can be explained by mesenchyme-expressed ligand failing to activate Fgrf2(IIb) in adjacent epithelium. For the limb and the lung, expression of the ligand (Fgf10) and receptor (Fgrf2(IIb)) are well documented in adjacent cell lineages, and fetuses deficient for Fgf10 show a similar phenotype to the Fgrf2(IIb)−/− mice. In the limb, there is compelling evidence of signalling by Fgf10 in the lateral plate mesoderm to Fgrf2(IIIb) in the overlying ectoderm, which leads to formation of the AER (Ohuchi et al., 1997). The AER then acts as an important centre for signalling to the underlying mesoderm thus establishing the progress zone for continued growth and patterning. The data herein indicate that AER formation is dependent upon Fgrf2(IIIb) and its absence causes both the presumptive limb ectoderm and mesoderm to undergo extensive apoptosis.

An analogous explanation could also account for the early failure of lung and anterior pituitary development in the Fgrf2(IIIb)−/− mice. Fgf10 is present in the lung mesoderm prior to budding of the bronchi from the tracheal ectoderm, and in the infundibulum during pituitary development. For the lung, Fgrf2(IIIb) is known to be present in the responding ectoderm. Furthermore, the importance of this signalling mechanism, not only for the limb, lung and anterior pituitary, but also for several other tissues and structures is suggested by defects found in the skin, salivary gland, tooth, and inner ear.

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