The IIIc alternative of Fgfr2 is a positive regulator of bone formation

Vereragavan P. Eswarakumar¹, Efrat Monsonego-Ornan², Mark Pines², Ileana Antonopoulou³, Gillian M. Morriss-Kay³ and Peter Lonai¹,*

¹Department of Molecular Genetics, The Weizmann Institute of Science, Rehovot Israel  
²Institute for Animal Science, The Volcani Center, Beit Dagan, Israel  
³Department of Human Anatomy and Genetics, University of Oxford, Oxford, UK  
*Author for correspondence (e-mail: peter.lonai@weizmann.ac.il)

Accepted 17 May 2002

SUMMARY

Fibroblast growth factor receptor type 2 (FGFR2) plays major roles in development. Like FGFR1 and FGFR3, it exists as two splice variants, IIIb and IIIc. We have investigated in the mouse the function of FGFR2IIIc, the mesenchymal splice variant of FGFR2. Fgfr2IIIc is expressed in early mesenchymal condensates and in the periosteal collar around the cartilage models; later it is expressed in sites of both endochondral and intramembranous ossification. A translational stop codon inserted into exon 9 disrupted the synthesis of FGFR2IIIc, the epithelial Fgfr2 variant. The recessive phenotype of Fgfr2IIIc mutant mice was characterized initially by delayed onset of ossification, with continuing deficiency of ossification in the sphenoid region of the skull base. During subsequent stages of skeletogenesis, the balance between proliferation and differentiation was shifted towards differentiation, leading to premature loss of growth, synostosis in certain sutures of the skull base and in the coronal suture of the skull vault, with dwarfism in the long bones and axial skeleton. The retarded ossification was correlated with decrease in the localized transcription of the osteoblast markers secreted phosphoprotein 1 (Spp1) and Runx2/Cbfa1. A decrease in the domain of transcription of the chondrocyte markers Ihh and PTHrP (Pthlh) corresponded with a decrease in their transcripts in the proliferative and hypertrophic chondrocyte zones. These results suggest that Fgfr2IIIc is a positive regulator of ossification affecting mainly the osteoblast, but also the chondrocyte, lineages. This role contrasts with the negative role of Fgfr3, although recent reports implicate FGF18, a ligand for FGFR3IIIc and FGFR2IIIc, as a co-ordinator of osteogenesis via these two receptors.

Key words: Transcriptional alternatives, FGF, Endochondral ossification, Craniosynostosis, Gene targeting, Mouse

INTRODUCTION

Three of the four fibroblast growth factor receptors, FGFR1, 2 and 3, express splice variants, which alternatively use exon 8 or 9 to encode the C-terminal half of immunoglobulin-like loop III in the extracellular ligand-binding domain of the receptor (for review, see Johnson and Williams, 1993). The two alternative variants each display unique binding specificity. IIIb-type receptors, which use exon 8, bind FGFs that are localized mostly in the mesenchyme, whereas IIIc-type receptors that use exon 9 preferentially recognize epithelial FGFs (Ornitz et al., 1996). Their localization follows the same regulatory logic. Fgfr2IIIb is expressed in the surface ectoderm and in the endothelial lining of internal organs from early organogenesis to adult life, while Fgfr2IIIc is transcribed in the paraxial and lateral mesoderm, in the limb bud and branchial arch mesenchyme and later in muscle and other mesenchymal tissues, including the periphery of bone forming cartilage models (Orr-Urtreger et al., 1993). This reciprocal mesenchymal and epithelial expression of the receptor alternatives and their respective ligands forms the basis of the paracrine FGF-FGFR2 interactions that make major contributions to vertebrate development and growth. Although localized expression of the splicing alternatives of Fgfr1 and Fgfr2 has been studied in less detail, they too appear to conform to an equivalent coordinated expression and localization of their two splice variants (Kettunen et al., 1998; Beer et al., 2000).

Epithelial-mesenchymal interactions are fundamental to organogenesis; understanding the function of FGFR variants in the cross-talk between embryonic tissues is therefore of considerable importance. Exon-specific gene targeting revealed that the activity of Fgfr1 during late gastrulation (Deng et al., 1994; Yamaguchi et al., 1994) is mostly due to its IIIc alternative (Partanen et al., 1998). Recent results imply that the IIIb alternative of Fgfr1 may be active in skin development, serving as a receptor for the mesenchymal FGF10 ligand (Beer et al., 2000). The role of Fgfr2IIIb has been studied in detail (De Moerlooze et al., 2000; Revest et al., 2001) (our unpublished results). Loss of Fgfr2IIIb abrogates limb outgrowth with multiple defects in branching morphogenesis. This phenotype is similar to the null mutation of Fgfr2 after rescuing its placentaion defects (Arman et al., 1999), as well as to loss of function mutation of Fgf10 ( Sekine et al., 1999).
We have analysed the functional activity of Fgfr2IIIc, the second alternative product of Fgfr2, which utilizes exon 9. This splice variant is expressed in the skeletogenic mesenchyme (Orr-Urtreger et al., 1993), and activating mutations in exon 9 of the human FGFR2 gene are associated with craniosynostosis (Wilkie, 1997). To create a loss-of-function phenotype, we introduced a point mutation into exon 9 of Fgfr2, which caused a frame-shift and created a translational stop codon, without influencing the expression of Fgfr2IIIB. Our data show that loss of Fgfr2IIIc results in a recessive viable phenotype with craniosynostosis and retarded development of the axial and appendicular skeleton, causing dwarfism and misshapen skull. We show that the normal expression of Spp1, Cbfa1, Ihh and PTHrP (Pthlh - Mouse Genome Informatics) requires Fgfr2IIIc, consistent with the role of this receptor in maintaining the normal rate of bone formation. These results demonstrate that Fgfr2IIIc fulfills a positive role in bone development, in contrast to negative regulation by Fgfr3 (Colvin et al., 1996; Deng et al., 1996).

MATERIALS AND METHODS

Gene targeting
A previously described genomic fragment including exons 7, 8, 9 and 10 of Fgfr2 (Arman et al., 1998) was used for site-directed mutagenesis, using the ‘Gene Editor’ kit (Promega) according to the manufacturer’s instructions. To inactivate exon 9, the IIIc-specific exon of Fgfr2, dGTP was inserted at codon 333 (58 nucleotides after the beginning of exon 9, considering the Fgfr2IIIc cDNA), which created a stop codon five nucleotides downstream and produced a HindIII site two nucleotides upstream from the site of insertion (Fig. 1A,B). Similarly, to inactivate exon 8, the IIIb-specific exon of Fgfr2, dGTP was inserted at codon 344 (93 nucleotides after the beginning of Exon 8, considering the IIIb cDNA), creating a stop codon five nucleotides downstream and producing a HindIII restriction site two nucleotides upstream from the site of insertion. A ‘floxed’ neomycin resistance gene was inserted into intron 9 of both lines and into a control line (the only change made). Mutagenized fragments were ligated into the “Osudpdel” vector (a gift from Professor Oliver Smithies, University of North Carolina, Chapel Hill). Homologous recombination into R1 ES cells was as described previously (Arman et al., 1998). Mutagenized and wild-type ES cells were analysed by Southern blot hybridisation to confirm the predicted sizes of the 5’ and 3’ restriction enzyme fragments (Fig. 1C). The neo cassette was removed from all strains by mating to an early deleter strain (Lallemand et al., 1998).

Histology and skeletal preparations
Cryostat or paraffin-embedded sections were stained with Haematoxylin and Eosin. Alizarin stained skeletal preparations were made according to the method of Kaufman (Kaufman, 1992). Cells in the hypertrophic and proliferating chondrocyte columns were counted over three mutant and three wild-type tibiae, using three consecutive sections of each. In situ hybridization
Whole-mount in situ hybridization of E16.5 fetal heads was carried out as described previously (Iseki et al., 1999). Radioactive in situ hybridization and the Fgfr2IIIB and IIIc probes were as described by Orr-Urtreger et al. (Orr-Urtreger et al., 1993). The probe for Runx2/Cbfal was a gift from Dr G. Karsenty (M. D. Anderson Cancer Center, Houston, Texas), for Ihh, from A. McMahon (Dept. of MCB, Harvard University, Boston, Mass.), for PTHrP from M. Kronenberg (Mass. General hospital, Boston, Mass.) and for Spp1 from B. Hogan (Vanderbilt University, Memphis, Tenn.).

BrDU assay
Pregnant females were injected with a 10 mg/ml solution of BrdU (Roche), at 100 μg/g body weight. Embryos were fixed in Bouin’s fluid and embedded in paraffin. Alternate tissue sections were stained with Mallory’s trichrome for histology; the others were incubated with anti-BrdU antibody (Roche) and visualized with biotin-conjugated goat anti-mouse IgG (Vector) followed by peroxidase reaction (brown colour for BrdU-positive nuclei). These sections were counter-stained with Haematoxylin (blue nuclei). Analysis was carried out using a Kontron (Zeiss) KS400v3 automatic image analyser. A defined area including the osteoid (unmineralized matrix) of the frontal and parietal bones in the coronal sutural region, together with the preosteoblasts and osteoblasts, but excluding adjacent non-skeletogenic membrane tissues, was outlined and measured. Within equivalent areas of each of 10 wild-type and 10 mutant sections, BrdU-positive and total nuclear area was assessed by setting the detection levels for brown only or brown plus blue. Ratios were calculated for each pair of measurements and the ratios for wild-type and mutant embryos compared by the Student’s t-test. For each defined area, BrdU uptake was also assessed by counting the BrdU-positive cells; these counts were separately compared by the Student’s t-test.

Photography
A Zeiss Axioplan, a Leitz macroscope, or a Nikon DXM1200 microscope with a CCD camera were used.

RESULTS

Targeted disruption of Fgfr2IIIc
Three different mouse strains were produced. The two experimental lines carry translational stop codons introduced by site-directed mutagenesis into one of the two alternatively spliced exons, either exon 8 (IIIb) or exon 9 (IIIc) and a neomycin resistance cassette was inserted into intron 9; the control line carried only the “floxed” neo cassette. After removing the neo cassette, all three heterozygotes were normal and fertile. The control homozygous showed a normal phenotype, indicating that the residual loxP site had no effect on development. The Fgfr2IIIB+/- homozygote was perinatal lethal, with limb, submaxillary gland and lung agenesis, as described recently by others (De Moerloose et al., 2000; Revest et al., 2001).

Mice homozygous for the Fgfr2IIIc mutation (Fig. 1), are viable and fertile. They are distinguished by small size and abnormally shaped head (see later). To evaluate the specificity of the Fgfr2IIIc loss-of-function phenotype, it was important to know whether the mutation affects the expression and splicing of the alternative Fgfr2IIIB variant. In situ hybridization of sections prepared from E12.5 embryos detected Fgfr2IIIB transcripts in the surface ectoderm of the developing wild-type and Fgfr2IIIc+/- limb (Fig. 2A-D). At E14.5, Fgfr2IIIB transcripts were detected in the perichondrium of prevertebrae and ribs, and in the branching epithelium of the lungs in both wild-type and Fgfr2IIIc+/- embryos (Fig. 2E-H). We therefore conclude that the point mutation in exon 9 results in a phenotype specific for the IIIc splice variant of Fgfr2.

Fgfr2IIIc expression was, as described previously (Orr-
Utrreger et al., 1993), mainly in the mesenchymal tissues of the organs affected by the mutation. At E12.5 these included skeletal condensations prior to cartilage model formation (Fig. 3A,B). By E14.5, the Fgfr2IIIc transcripts were down-regulated in the cartilage models except for the perichondrium, as this tissue begins to transform into the periosteal collar of bone, and in the region of the future epiphyseal plate (Fig. 3C,D). In endochondral bones it was expressed in the peristeum and in regions of osteogenesis (Fig. 3E,F). These observations suggest roles for Fgfr2IIIc during initial specification of skeletogenic mesenchyme, but thereafter it is confined to the osteoblast rather than the chondrocyte lineage.

Growth and ossification defects in the Fgfr2IIIc–/– mutant

From the second postnatal week, the mutant can be distinguished from its littermates by its domed skull, slightly bulging eyes and shortened and sometimes bent facial area (Fig. 4A). Skeletal preparations at P14 (Fig. 4B) reveal that although of reduced size, bones of the axial and appendicular skeleton are of normal shape and proportions. The mutant skull was however much shorter than the wild type; both facial and neurocranial regions were affected, with down-curved nasomaxillary region, prognathia of the lower incisors and a rounded skull vault. Growth during the postnatal period, as indicated by weight, is 40-50% less in mutants than in their wild-type littermates (Fig. 4C). Some have now been alive for over a year, but have stayed significantly smaller than their littermates. Initially we assumed that the small size of the mutant was caused by starvation due to feeding difficulties because of overgrowth of the lower incisors. This seems unlikely, since we routinely cut these teeth back as early as possible; furthermore slower weight gain was already evident in homozygous mutant pups before the incisors erupted.

The first signs of general ossification defects were observed in Alizarin-stained skeletal preparations of E14.5 wild-type and mutant fetuses (Fig. 5A,B). The onset of ossification was delayed in the mutant, so only the earliest bones to undergo ossification were stained, i.e. the mandible, clavicle, scapula (blade), humerus and proximal parts of the upper ribs. The delay affected both dermal and endochondral components of the skull vault and skull base, and there was no ossification of the hindlimbs or vertebrae. Investigation of isolated vertebrae at P14 revealed that the vertebral arches were not yet fused in the dorsal midline in the mutant (Fig. 5C-F). It follows that Fgfr2IIIc is required for the normal timing of mineralization of endochondral bones, hence delayed osteogenesis and not inadequate feeding was responsible for dwarfism in Fgfr2IIIc–/– mice.

Fig. 1. Targeted disruption of the Fgfr2IIIc transcriptional alternative. (A) Genomic structure and targeting events; exons are shaded, with the exon number above, and the protein domain name underneath. c and g, 5¢ and 3¢ probes respectively; B, BamHI; H, HindIII; RI, EcoRI; S, SacI; TM, transmembrane exon; #, site of point mutation. (B) DNA sequence of the region used for site-directed mutagenesis, showing the newly formed HindIII site and the translational stop codon (*). (C) Southern blot analysis of the homologous recombinant ES cells, probed with the 5¢ and 3¢ fragments designated in A.

Morphogenesis of the endochondral skull base

The anatomical basis of abnormal skull formation of the mutant was investigated in Alizarin-stained skeletal preparations. Fetal skulls from E16.5 to P14 revealed reduced
size of the bones in the mutant skull base (Fig. 6). By E18.5 the suture between the basioccipital and the exoccipital bone started to fuse, and by P3 the basioccipital and basisphenoid bones were in contact. By P14, mineralization was greatly reduced, especially in the sphenoid bone. All of the skull base sutures were open in the wild-type skull at this stage. In the mutant, sutures between the basioccipital and exoccipital bones (Fig. 6), and between the exoccipital and supraoccipital bones (not shown), were fused; fusion had also begun in the basioccipital-basisphenoid suture. The basisphenoid-presphenoid suture was abnormally positioned under the bony palate, because of shortening of the mutant skull base.

Measurement of the skull base bones revealed that most of the shortening was due to reduced growth of the middle (sphenoid) region. The wild type:mutant ratio of bone lengths on the P14 specimen illustrated in Fig. 6 is 1:0.95 from the outer border of the exoccipital bone to the basioccipital-

sphenoid suture, but 1:0.55 from this suture to the caudal border of the maxilla. This is also evident as reduced distance between the otic capsule and the caudal border of the tooth row on each side.

**Differentiation and growth of the skull vault**

Osteopontin, which is encoded by the gene secreted phosphoprotein 1 (Spp1), is one of the major non-collagenous bone matrix proteins, produced by osteoblasts and osteoclasts. It is copiously expressed by mineralized bone and is involved in bone remodelling. Hence Spp1 expression is a good indicator of osteogenesis and can be used to detect the developing bone domains in the skull vault by means of whole-mount in situ hybridisation (Iseki et al., 1997). At E16.5, Spp1 expression revealed a delay in expansion of the frontal bone domains towards the midline to form the metopic suture; the parietal and interparietal domains were normal, but the nasal bone domains were undetectable (Fig. 7A,B). In alizarin-stained specimens at P3 the pattern of skull vault bones was not detectably different in mutant and wild-type pups (not shown). By P14, the metopic, sagittal and lambdoid sutures of both mutant and wild-type skulls were unfused, but the mutant coronal suture showed partial (medial) or complete bony fusion in some
specimens (Fig. 7C-F). These observations suggest that there is a catch-up following the late onset of ossification seen in E14.5 specimens, and that this more rapid rate of ossification in the mutants continues as premature loss of the coronal suture in at least some pups by P14. Except for the coronal suture, morphogenesis of the skull vault was less affected than that of the skull base.

To obtain information on the effects of loss of Fgfr2IIIC function in the coronal suture at earlier stages, the rate of proliferation was investigated by BrdU incorporation at E14.5, E16.5 and P1 (Fig. 8). Differentiation was assessed in parallel sections by Mallory trichrome stain to reveal secreted bone matrix. Cell counts and nuclear area measurements (see Materials and Methods) gave the same results following statistical analyses. No differences in BrdU uptake were detected at E14.5 (P>0.5), when the characteristic sutural organisation is first detectable (Johnson et al., 2000). At E16.5 there were fewer BrdU-positive cells in the sections of mutant tissue (P<0.001). At P1, groups of BrdU-positive cells were detected within the mesenchymal tissue distal to the borders of the frontal and parietal bones. In the mutant, very few BrdU-positive cells were detected (P<0.001), the expanded mesenchyme of the sutural region was lost, and the bone edges were blunt and irregular instead of being slender and tapering in profile. Mallory staining of sections showed normal unmineralized bone matrix structure in the coronal suture at E14.5 (not shown), but by P1 the frontal and parietal edges lacked the normal fine-edged overlapping structure (Fig. 8G,H).

These observations on the skull vault indicate that after an initial delay at E14.5 (Fig. 5), differentiation and

---

Fig. 4. Dwarfism and abnormal skull shape in homozygous Fgfr2IIIC mutants. (A) Live P14 wt and mutant mice. (B) Alizarin Red skeletal staining of P14 wild-type and mutant mice, showing overall small size, rounded skull, lower incisor overgrowth and shorter facial area in the mutant. (C) Growth curves demonstrating 40-50% growth retardation in the mutant.

Fig. 5. Delayed onset of ossification in the Fgfr2IIIC–/– mutant: Alizarin Red S staining of skeletal preparations. (A,B) E14.5 embryos, showing delayed mineralization of the whole skeleton. (C-F) First and second cervical vertebrae at P14, showing incomplete vertebral arches in the mutant.
Fig. 6. Mineralization of the skull base. After the slight delay of ossification observed in the mutant at E16.5, sutures between exoccipital and basioccipital bones begin to close as early as E18.5. White arrows point to the basioccipital-exoccipital junction, where in the mutant precocious fusion takes place. Black arrows on the P14 mutant specimen indicate complete fusion of the basioccipital-exoccipital suture and contact at the basioccipital-basisphenoid suture; the vertical arrow indicates the abnormal position of the basisphenoid-presphenoid suture beneath the palate, due to shortening of the sphenoid area in the mutant. bo, basioccipital bone; pal, palate; bs, basisphenoid bone; eo, exoccipital bone; so, supraoccipital bone.

Fig. 7. Differentiation and mineralization of the skull vault. (A,B) Spp1 expression at E16.5 shows delayed formation of the metopic suture and is absent in the nasal bones in the mutant (arrow). (C–F) Alizarin-stained P14 skeletal preparations, showing premature fusion of the medial part of the coronal suture in the Fgfr2IIIc mutant. cs, coronal suture; ls, lambdoid suture; ss, sagittal suture.

Fig. 8. Decreased cell proliferation in the coronal suture of Fgfr2IIIc−/− fetuses during skull vault growth. (A,B) Levels of BrdU incorporation are normal at E14.5. (C,D) Numbers of BrdU-labelled cells are reduced at E16.5. (E,F) At P1, only a few BrdU-positive cells are observed in the mutant. (G,H) Parallel Mallory-stained P1 sections showing the ossification fronts. al, alisphenoid cartilage; f, frontal bone; p, parietal bone. Scale bar: 100 μm.
mineralization of the skull vault becomes normal except for the coronal suture, where the proliferating osteoblasts are prematurely lost (Fig. 8), leading to partial or complete synostosis by P14 (Fig. 7C-F). Deficiency of the coronal suture as a growth centre may contribute to doming of the skull, but deficient growth of the skull base is likely to be a more significant cause of this feature.

The effects of loss of Fgfr2IIIc function on endochondral differentiation

During endochondral bone formation and growth, proliferating chondrocytes in the diaphysis of the cartilaginous model at early stages, and in the growth plates throughout the period of bone growth, undergo hypertrophy and finally apoptosis. They produce a framework of cartilage matrix, which forms a template for the deposition of bone matrix proteins by invading osteoblasts. The first ossification is in the perichondrium, which is transformed into the periosteal collar of bone around the diaphysis, before the primary (diaphyseal) ossification center has formed. To gain insight into the effects of loss of Fgfr2IIIc function on endochondral bone differentiation, we investigated gene expression in the skull base at E18.5 and P7, and in the tibial growth plate at P7 and P14. Transcript levels of the osteogenic differentiation marker Spp1, which is expressed in the differentiating osteoblasts, were reduced in the regions of ossification of both the skull base (Fig. 9A-D) and the tibia at P14 (Fig. 9E,F), confirming that Fgfr2IIIc affects endochondral bone formation in both the skull base and the long bones.

In the P7 tibial growth plate (Fig. 10A,B) there is a considerable narrowing of the hypertrophic chondrocyte layer in the mutant compared to wild type. This observation was confirmed by cell counts of the chondrocyte columns, which also revealed an effect on the proliferative chondrocyte zone (Fig. 10C).

The effects of loss of Fgfr2IIIc function on endochondral bone growth

Gene expression patterns in the skull base were investigated in the cartilaginous region that underlies the pituitary gland, including the adjacent ossified areas of the basioccipital and basisphenoid bones. This region comprises a central area of resting chondrocytes flanked by proliferating chondrocyte zones, hypertrophic chondrocyte zones, and trabecular bone (ossification zone), in a central to peripheral sequence. Runx2/Cbfa1 expression was investigated in sagittal sections of E18.5 fetuses (Fig. 11A-D). Transcripts were detected in the
ossification zone, and within and around the bony trabeculae of the basioccipital bone (Fig. 11B), with much reduced transcript levels in the mutant (Fig. 11D). Fgfr2IIIc signaling is therefore required for the normal expression of both Runx2/Cbfa1, which is detectable in early osteoblasts (Ducy et al., 1997) and Spp1, which is expressed in mature osteocytes during mineralization.

Chondrocyte differentiation is orchestrated by a reciprocal regulatory loop between Indian hedgehog (Ihh) and the parathyroid hormone-related peptide (PTHrP/Pthlh) (Kronenberg et al., 1997; St-Jacques et al., 1999; Chung et al., 2001). After formation of the primary ossification centre, PTHrP expression is detected in the osteoblasts of the bone collar and in the trabecular bone of the ossification centre (Lee et al., 1995). We therefore investigated the effect of the Fgfr2IIIc mutation on PTHrP and Ihh expression in the postnatal (P7) skull base and tibial growth plate (Fig. 11E-P). Expression of PTHrP in the periosteal bone collar, the osteogenic front and the trabecular bone of the P7 skull base was greatly reduced in the mutant (Fig. 11F,H). Accumulation of Ihh transcripts in the pre-hypertrophic and hypertrophic chondrocyte layer of the skull base (Fig. 11J,L) and tibial growth plate (Fig. 11N,P) was also reduced, albeit to a smaller extent. The decreased Ihh signal in hypertrophic chondrocytes was in domain size rather than intensity, reflecting the decreased length of this zone (Fig. 10). These data suggest that loss of Fgfr2IIIc affects regulators of both osteocyte and chondrocyte differentiation during endochondral bone growth.

**DISCUSSION**

We analyzed the consequences of the loss of Fgfr2IIIc in the mouse by inserting a guanine residue, creating a translational stop codon in exon 9, which encodes the amino terminal half of the third Ig-like loop of Fgfr2. In a recent report,
Hajihosseini et al. (Hajihosseini et al., 2001) described the complete deletion of exon 9 of Fgfr2, including significant portions of the flanking introns. Their deletion caused a dominant lethal mutation with splice switch and induced ectopic expression of Fgfr2IIb, leading to severe visceral and skeletal abnormalities. This mutation is an interesting example of defects that may result from the inappropriate localization or abnormal binding specificity of the Fgfr2 alternatives. Such defects have been associated with Apert syndrome (Anderson et al., 1998; Oldridge et al., 1999; Yu and Ornitz, 2001). In contrast, the creation of a translational stop codon in exon 9, as described here, did not affect the localized transcription of the IIIb splice variant, which uses exon 8. We therefore conclude that the recessive, viable phenotype resulting from this mutation is specifically due to the loss of Fgfr2IIc.

Fgfr2IIIC<sup>+/−</sup> mutants displayed normal limb development, the reduced length of the limb bones being proportional to the reduced size of the whole skeleton. This phenotype is distinct from that caused by loss of the IIIb alternative. We prepared a similar frame shift mutation in exon 8 of Fgfr2IIb (V. P. E. and P. L., unpublished), which resulted in the limbless phenotype with defective branching morphogenesis as reported for Fgfr2IIIB<sup>+/−</sup> embryos by Revest et al. (Revest et al., 2001). Fgfr2IIb is localized in the apical ectodermal ridge (AER) of the limb bud (Orr-Urtreger et al., 1993); this receptor isoform interacts with FGF10 ligand in the progress zone mesenchyme (Ohuchi et al., 1997; Xu et al., 1998), whereas Fgfr2IIIC transcripts are detectable in the limb bud mesenchyme. The normal limb development observed in the Fgfr2IIIC loss of function mutation suggests that this receptor may not be the partner of AER-derived FGFs. The IIIC variant of FGFR1 is a more probable receptor for these growth factors, since mutation of this receptor isoform results in a hypomorphic limb phenotype (Partanen et al., 1998).

A number of mutations of the IIIC alternative of FGFR2 and FGFR1 are associated with different types of human craniosynostosis syndromes. It is generally accepted that most FGFR mutation-derived dominant human craniosynostoses are due to gain of function resulting from ligand-independent signaling (for review, see Wilkie, 1997). Craniosynostosis in our mutation was distinguished from the human gain of function phenotypes by its predominant manifestation in the endochondral skull base, and by its recessive inheritance.

The occurrence of synostosis of the coronal suture in both the murine Fgfr2IIIC loss-of-function mutation demonstrated here and in human gain-of-function mutation in the same splice variant, such as the Crouzon syndrome, appears at first sight to be paradoxical, but is a logical outcome of the mechanism of activity of this receptor. Functional studies in the mouse revealed that Fgfr1 and Fgfr2 play reciprocal roles in maintaining the proliferation-differentiation balance in osteoprogenitor cells of the coronal suture, and are expressed respectively in differentiating and proliferating cells (Iseki et al., 1999). The proliferation-differentiation balance is maintained by differential effects of high and low levels of FGF-FGFR signaling. Adding exogenous FGF2 ligand to the developing coronal suture results in up-regulation of Fgfr1 and increased osteogenic differentiation, with concomitant down-regulation of Fgfr2 and decreased osteogenic cell proliferation.

The progressive loss of osteogenic proliferating cells in the coronal suture in the absence of Fgfr2IIIC, as reported here, is consistent with this model. Fgfr2IIIC also plays a role in endochondral ossification. The onset of mineralization was retarded in our mutants, and the growth of the skull base and axial and appendicular skeletons was reduced. This outcome was associated with decreased areas of both proliferating chondrocytes and ossification zones in these endochondral bones, leading to premature loss of skull base sutures and smaller than normal long bones and vertebrae. Consistent with these observations, we found a significant decrease in the accumulation of transcripts characteristic of chondrocytes and osteoblasts, suggesting that both require Fgfr2IIIC function. This transcriptional alternative is expressed in the mesenchymal condensations that form the cartilage models of endochondral bones. Later, its transcription shifts to the perichondrium as it transforms into the periosteal bone collar at the onset of osteogenesis, and then in the developing trabecular bone of the ossification zone. It is not clear how Fgfr2IIIC in the ossification zone influences chondrocyte proliferation in the growth plate. It is possible that this effect is the result of events occurring during its early mesenchymal expression, when Fgfr2IIIC could affect common precursors of both lineages and thus influence the size of cartilage models, the onset of differentiation and the long term process of endochondral bone growth. Alternatively, the primary target of Fgfr2IIIC may be the osteocyte lineage through activation of Runx2/Cbfa1, which has a major role in osteogenesis (Ducy et al., 1997) but also influences chondrogenesis (Kim et al., 1999; Takeda et al., 2001).

Recent publications by Liu et al. (Liu et al., 2002) and Ohbayashi et al. (Ohbayashi et al., 2002) investigated the loss-of-function phenotype of Fgfr18. Both papers report that the phenotype resembles the Fgfr3 loss-of-function phenotype resulting in extended long bone growth, but also delayed ossification with decreased expression of osteogenic markers. They suggest that FGF18 signaling co-ordinates chondrocyte and osteoblast differentiation. The evidence we present here suggests that the FGRF2IIIC receptor, which together with FGR3IIIIC binds FGF18, may contribute to this co-ordinated regulation of osteogenesis.

The expression pattern of Fgfr2IIIC in developing endochondral bones is different from those of Fgfr1 and Fgfr3. Fgfr1 is expressed in the perichondrium during early bone formation, but is later transcribed by proliferating and hypertrophic chondrocytes of the growth plate (Orr-Urtreger et al., 1991; Peters et al., 1992; Delezoide et al., 1998). Fgfr3 transcription localizes to the resting and proliferating chondrocyte layers (Deng et al., 1996) and its loss results in long bone overgrowth with massive extension of the proliferating chondrocyte layer (Peters et al., 1993; Deng et al., 1996; Colvin et al., 1996). Gain-of-function mutations of Fgfr3 create the opposite phenotype. They cause achondroplasia in man and their molecular effects include repression of Ihh and PTHrP signaling (Naski et al., 1998) and activation of cell cycle inhibitors (Li et al., 1999; Sahni et al., 1999). This is in contrast to the dwarfism and decrease of Spp1, Ihh, PTHrP and Chafal expression in our Fgfr2IIIC loss-of-function mutation. This contrast is emphasised in a gain-of-function mutation of Fgfr2IIIC with a C342Y amino acid replacement in exon 9, in which Chafal/Runx2 and Spp1 transcription is activated (V. P. E., G. M. M.-K. and P. L., unpublished).
Comparison of the role of FGFRs in bone development suggests that the rate of proliferation and differentiation of skeletogenetic precursors involves cooperation between different FGFRs. In the coronal suture, synostosis can result from gain-of-function mutations of FGFR1, FGFR2 and FGFR3 (Bellus et al., 1996). Although the splice variants of Fgfr1 and Fgfr3 are yet to be studied, these genes show specific expression patterns and function in the coronal suture (Iseki et al., 1999; Orr-Urtreger et al., 1993; Delezoide et al., 1998) and in developing dental tissue (Kettunen et al., 1998).

The present results extend these data, suggesting a balanced cooperation between the negative regulation due to Fgfr3 and the positive control exerted by Fgfr2IIIc. The data of Liu et al. (Liu et al., 2002) and Ohbayashi et al. (Ohbayashi et al., 2002), discussed above, suggest that this co-ordination of chondrocyte and osteoblast differentiation is mediated through FGFR8. All three of the alternatively spliced Fgfr genes are active during early osteogenesis and influence the expression of multiple bone development genes. Hence, gene expression and lineage decisions required for osteogenesis may depend on their balanced cooperation. The mode of this control, whether conveyed by transcriptional regulation and/or by indirect means involving the bone matrix, remains to be elucidated.

This study was supported by grants from the Israel Science Fund and from Prochon Biotechnology, Ltd. to P. L., and a grant from Action Research to G. M. M.-K. I. A. is in receipt of a research studentship from the Anatomical Society of Great Britain and Ireland.

REFERENCES


Iseki, S., Wilkie, A. O. M., Heath, J. K., Ishimaru, T., Eto, K. and Morriss-Kay, G. M. (1997). Fgfr2 and osteopontin domains in the developing skull vault are mutually exclusive and can be altered by locally applied FGFR2.


