Skeletal development is regulated by fibroblast growth factor receptor 1 signalling dynamics

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Summary

Ligand-dependent signalling pathways have been characterised as having morphogen properties where there is a quantitative relationship between receptor activation and response, or threshold characteristics in which there is a binary switch in response at a fixed level of receptor activation. Here we report the use of a bacterial artificial chromosome (BAC)-based transgenic system in which a hypermorphic mutation has been introduced into the murine Fgfr1 gene. These mice exhibit cranial suture and sternal fusions that are exacerbated when the BAC copy number is increased. Surprisingly, increasing mutant BAC copy number also leads to the de novo appearance of digit I polydactyly in the hind limb and transformations of the vertebrae. Polydactyly is accompanied by a reduction of programmed cell death in the developing hind limb. Candidate gene analysis reveals downregulation of Dkk1 in the digit I field and upregulation of Wnt5a and Hoxd13. These findings show that Fgfr1-mediated developmental pathways exhibit differing signalling dynamics, whereby development of the cranial sutures and sternum follows a morphogen mode, whereas development of the vertebral column and the hind limbs has threshold signalling properties.

Supplemental data available online

Key words: Fgf, Signalling, Skeleton

Introduction

Although much is known about the connectivity of receptor-mediated developmental signalling pathways, relatively little is known about the relationship between the dynamics of receptor activation and the ensuing response. Freeman and Gurdon have drawn a distinction between ‘morphogen signalling’ – in which the response is quantitatively linked to the concentration of activating ligand (and therefore activated receptors) – and ‘threshold’ signalling in which the response is triggered in a binary manner at a pre-set level of receptor activation. Here, we report the use of a bacterial artificial chromosome (BAC)-based transgenic system in which a hypermorphic mutation has been introduced into the murine Fgfr1 gene. These mice exhibit cranial suture and sternal fusions that are exacerbated when the BAC copy number is increased. Surprisingly, increasing mutant BAC copy number also leads to the de novo appearance of digit I polydactyly in the hind limb and transformations of the vertebrae. Polydactyly is accompanied by a reduction of programmed cell death in the developing hind limb. Candidate gene analysis reveals downregulation of Dkk1 in the digit I field and upregulation of Wnt5a and Hoxd13. These findings show that Fgfr1-mediated developmental pathways exhibit differing signalling dynamics, whereby development of the cranial sutures and sternum follows a morphogen mode, whereas development of the vertebral column and the hind limbs has threshold signalling properties.

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Fgfr2 ‘linker’ mutations have been shown to exhibit increased affinities for specific Fgf ligands (Anderson et al., 1998; Ibrahimii et al., 2001) because of the additional receptor/ligand contact site introduced by the missense substitution. This finding suggests that Apert/ Pfeiffer-type linker domain substitutions result in receptors that are activated at lower concentrations of ligand compared with their wild-type counterparts, and that exhibit quantitatively distinct ligand-dependent signalling dynamics. As receptor activation is initiated by ligand-mediated receptor dimerisation (reviewed by Schlessinger, 2000), only mutant receptor homodimers exhibit mutant signalling dynamics. This consideration predicts that the phenotypic consequences of mutant receptor signalling will be dictated by the ratio of mutant to wild-type receptors, as elevating the ratio will favour the formation of mutant homodimer complexes in the presence of appropriate ligands. Thus, varying the expression of mutant receptors provides a means to study the consequences of quantitative changes in Fgfr signalling dynamics.

Here we employ a novel bacterial artificial chromosome (BAC)-based transgenic system (Lalioti and Heath, 2001) to dissect the molecular consequences of mutant Fgfr action in vivo. We have introduced into the mouse germ line a BAC encoding the entire mouse Fgfr1 gene that harbours a single nucleotide substitution corresponding to the human Pfeiffer syndrome mutation Pro252Arg (Muenke et al., 1994). We find that, in contrast to BACs harbouring silent single nucleotide substitutions, the presence of the mutant BAC transgene yields skeletal defects that involve both membranous and endochondral modes of ossification, resembling those seen in human Pfeiffer patients. Doubling the BAC gene copy number – thereby incrementally elevating signalling through mutant receptors – yields an increase in the severity of the ossification defects, and an unexpected de novo appearance of pre-axial polydactyly of the hindlimbs and homeotic transformation of the vertebrae. These findings demonstrate the existence of a Fgfr1 signalling threshold governing the development of digit I and vertebral patterning. This study also shows – as predicted by Freeman and Gurdon (Freeman and Gurdon, 2002) – that a single signalling pathway can exhibit both morphogen and ligand-dependant signalling dynamics. As receptor activation is regulated by the level of receptor stimulus, we conclude that Apert/ Pfeiffer-type substitutions result in receptors that are activated at lower concentrations of ligand compared with their wild-type counterparts, and that exhibit quantitatively distinct ligand-dependent signalling dynamics.

Materials and methods

Isolation and characterisation of a Fgfr1 BAC clone

A mouse library [RPC-22 filters, from BacPac Resources (Osoegawa et al., 2000)] was probed with a digoxigenin-labelled Fgfr1 cDNA, generated by PCR using primer sets: exon 1 (1L), 5'-GTGAAATATCCATGAGGTAC-3' and (2R), 5'CTCCTCCCGAAGGAGGTCATC-3'. Positive clones (HGM, Hinxton, UK) were then screened by three PCR reactions, using sets of primers whose products would cover the entire 21 exons of the mouse Fgfr1 gene. These primers were:

- exon 1 (1L) and (3R), 5'-CGAAGAGGAGGAGCCTTC-3';
- exon 7 (2L), 5'-AGATCTGGGGAGGGTCTAAG-3' and (2R), 5'-CTCAGTGGTGGGACAG-3';
- exon 19 (4L), 5'-ATACCGCTGGGACGGTACTC-3' and (1R), 5'-TCAAGCCGTGTTGAGTCCACTG-3'.

Mutant Fgfr1 fragments were generated by overlap PCR. Essentially, two complementary primers covering the region to be mutated were used in conjunction with a pair of external primers to produce two overlapping fragments using Pfu polymerase (Promega). The two fragments were then joined together in a second reaction using only the external primers. For the Pro252Arg mutation (BAC16) the complementary primer pairs were (16L) 5'-GAATCCGCGGCCGCACAAG-3' and (16R) 5'-AGGAGGAGGGGATATGTAACGTT-3' and, external primers were (21L) 5'-TGCACGTCTGTCCTTCTGCTG-3' and (21R) (see above). These products were then cloned into the BamHI-SalI sites of the pKOV-kan shuttle vector in preparation for modification of the BAC as described previously (Lalioti and Heath, 2001).

Generation and screening of transgenic animals

Modified BACs were prepared for pronuclear injection as described (Chrst et al., 1999; Lalioti and Heath, 2001), and injected as linearized DNA (0.3-3 ng/ul) into fertilized oocytes. Genomic tail DNA from offspring was subjected to the following PCR conditions: 1 cycle of 5 minutes at 94°C, 20 cycles of 20 seconds at 94°C, 20 seconds at 60°C (with 1°C decrease per cycle), 30 seconds at 72°C, 20 cycles of 20 seconds at 94°C, 20 seconds at 50°C, 30 seconds at 72°C; 1 cycle of 5 minutes at 72°C. Primer pairs used were exon 7 (3L): 5'-TGGACGTCTGGTCTCTTCTG-3' and (3R): 5'-CCTCATAAGGATAGTATG-3'. The PCR products were then digested for 2 hours with MspI (for BAC16) or PseI (for BAC15), and resolved on 3% high resolution agarose gels (Elchrom Scientific).

To test expression of the transgene, 100 ng of total liver RNA isolated using Trizol® Reagent (GibcoBRL) was subjected to One Step RT-PCR (Qiagen) using the primers (8L) 5'-ACCTACCGGTCGGATCGTTG-3' and (6R) 5'-CATTCTTTGTCGTCGTAAC-3', as per manufacturer recommendations.

Skeletal staining

Skeletons from embryos, new born or adult mice were stained with Alcian Blue and/or Alizarin Red as described by McLeod (McLeod, 1980). A full listing of the animals examined with phenotypic annotation is provided in supplementary Tables S1 and S2.

Analysis of BAC-transgene copy number and sites of integration by fluorescence in situ hybridisation (FISH)

FISH analysis was carried out as described by Buckle and Rack (Buckle and Rack, 1993). Essentially, chromosomes were isolated from splenic cells, previously cultured for 48 hours in the presence of lipopolysaccharide (Triman et al., 1975), and hybridised with a mixture containing a bitoin 16-UTP-labelled murine Fgfr1 BAC probe and either Cy3-labelled mouse chromosome 8 paint or FITC-labelled mouse chromosome 4 paint (Cambio, Cambridge, UK). The hybridisation mixture contained 100 ng of biotinylated probe, 3 μg mouse COT-1 DNA (Invitrogen) and 20 μg salmon sperm DNA (Sigma), together with the appropriate amounts of paint, according to the manufacturer’s instructions. The biotinylated Fgfr1 probe was detected with either avidin Cy3.5 (Amersham Pharmacia) or avidin FITC (Vector Laboratories). Chromosomes were counterstained with 4',6-diamidino-2-phenylindole (DAPI) at 1.5 μg/ml and analysed using an Olympus BX-60 microscope equipped with a Pinkel filter wheel. Images were captured and analysed using a SenSys cooled CCD camera (Photometrics, Tuscon), and MacProbe version 4.3 software (Applied Imaging, UK).

Quantitative PCR analysis of BAC copy number/ transgene expression

Genomic DNA was amplified with primers (31L) and (34R) (see above; the reverse oligonucleotide was fluorescently labelled) using the following cycling conditions: 1 cycle of 10 minutes at 94°C; then 25 cycles of 30 seconds at 94°C, 30 seconds at 58°C and 30 seconds at 72°C; followed by 10 minutes at 72°C. The PCR products were digested for 1 hour with MspI and the fragments separated on an ABI 377 automated sequencer and analysed by GeneScan software (Applied Biosystems). cDNA (2 μl from a 40 μl first strand synthesis reverse transcription reaction using 2-5 μg total RNA) was amplified.
with primers (8L) and (6R) (see above; the reverse oligonucleotide was fluorescently labelled), using the same cycling conditions as for genomic DNA except the annealing temperature was 55°C. PCR products were digested for 2 hours with MspI and analysed as above.

**Whole-mount in situ hybridisation (WMISH)**

Whole embryos were isolated at appropriate stages of development from time-mated mice, counting noon of the day on which the vaginal plug was found as zero. Embryos were fixed overnight in 4% paraformaldehyde, dehydrated the following day through ascending concentrations of methanol/PBS containing 0.1% Tween-20, and stored until use in absolute methanol at –20°C. The genotype of each embryo (i.e. whether carrying low or high copies of the transgene) was verified by PCR (described above), using genomic DNA isolated from yolk sacs.

For WMISH, embryos were rehydrated in descending concentrations of methanol in PBS/Tween-20, treated with proteinase K, and hybridised at the appropriate temperatures (55-69°C) with sense (control) or anti-sense riboprobes generated from linearized plasmids containing the gene of interest (see Table S3 at http://dev.biologists.org/supplemental/). The protocol followed for WMISH was as previously described (Hajihosseini et al., 2001), with the exception that the colour development (NBT/BCIP) reaction was performed in the presence of polyvinyl alcohol (Sigma).

**Detection of apoptotic cell by neutral red staining**

Embryos were stained live as previously described (Hajihosseini and Heath, 2002). Briefly, soon after isolation, embryos were rinsed in freshly prepared 3% BSA/PBS and stained in the dark for 25 minutes (for E12.5) at room temperature in the same solution containing 0.05% Neutral Red. Excess dye was discarded and embryos were rinsed several times in 3% BSA/PBS, then in PBS and fixed for 15 minutes in 4% paraformaldehyde solution. Stained embryos were then photographed immediately.

**Results**

**Generation and characterisation of BAC transgenic mice**

A BAC construct harbouring a mutant Fgfr1 allele (Pro252Arg, hereafter referred to as BAC16; Fig. 1A) was made as described in the Materials and methods, and by Lalioti and Heath (Lalioti and Heath, 2001). As a control, a similar construct harbouring a silent Fgfr1 mutation (herein referred to as BAC15) was used. Transgenic lines were obtained by injection of the recombinant BAC DNA into fertilised eggs, with subsequent transfer of embryos to foster mothers. Founder mice carrying one or two copies of the transgenes (see below) were found to be viable and fertile, and were maintained on a C57-BL6 black background. Fluorescence in situ hybridisation (FISH) of splenic metaphase chromosomes derived from one particular line (designated BAC16/42) showed that the BAC transgene had integrated into the D3 region of chromosome 4 (Fig. 1B). The higher signal derived from the BAC transgene indicated that more than one copy had integrated. The results presented here are derived primarily from this line, although the phenotypes reported were seen in multiple independent lines. RT-PCR analysis on RNA isolated from mutant mice confirmed expression of the transgene in multiple tissues and showed this to be quantitatively comparable to the endogenous level of Fgfr1 expression (Fig. 1C; and data not shown). Quantitative PCR of genomic DNA confirmed that the BAC16/42 allele had integrated two copies of the BAC (data not shown). When single integration site BAC16 mice were intercrossed to generate homozygous integration alleles, the copy number – as expected from integration of two tandem copies – doubled to 4 (Fig. 1B,D). Quantitative PCR on selected tissue RNAs revealed that the expression levels of the mutant Fgfr1 gene were directly related to the BAC copy number and the endogenous wild-type gene copy number (data not shown). Examination of Fgfr1 expression at selected
embryonic stages did not reveal any evidence for misexpression of the transgene or endogenous Fgfr1. Expression of the mutant Fgfr1 alleles in BAC16 and BAC15 mice by these criteria therefore faithfully recapitulates the behaviour of the endogenous gene in both pattern and levels of expression. The relative ratio, and hence expression of mutant and wild-type receptors, can therefore be incrementally varied by intercrossing mice harbouring two copies of the BAC. Hereafter, the heterozygous BAC16 animals will be termed 2C and the homozygous BAC16 mice will be termed 4C.

Phenotype of 2C-BAC16 mice: defects in endochondral and membranous ossification

We began our analysis by examining the skeletons of BAC15 and 2C-BAC16 mice (Fig. 2; see also Tables S1 and S2 at http://dev.biologists.org/supplemental/). In contrast to sporadic phenotypes seen in BAC15s, which did not correlate with BAC transgene copy number, 2C-BAC16 mutants invariably presented defects in the cranium and the sternum, tissues that develop through two distinct modes of ossification: membranous and endochondral. In the skull, the BAC16 mutation yielded fusion (synostosis) of frontal bones through precocious ossification of the metopic (frontal) sutures. This was apparent as early as embryonic day 18.5 (E18.5) in the form of bony islands (Fig. 2A), but was more obvious at postnatal day 8, when a bony bridge across the metopic sutures was more apparent (Fig. 2F). Synostosis of sagittal sutures (separating the parietal bones) and coronal sutures (separating the parietal and frontal bones) was not observed. Further skull defects included shortening of the midface, curved maxilla, and fusion of joints separating the zygomatic arch bones (elements that make up the lower rim of the eye socket), which also developed through membranous ossification. Zygomatic fusion more frequently involved the anterior joint (separating the zygomatic branch of maxilla and the jugal bone) and occurred in a unilateral or bilateral fashion (Fig. 2D). Shortening of the face has been correlated with disparate growth of bones at the base of skull and fusion of their intervening joints (Eswarakumar et al., 2002). We could not detect fusion in joints separating the exoccipital, basi-occipital and basi-sphenoid bones (not shown), but in mice with facial curvature, we found a direct correlation between the phenotype (n=5/5) and unilateral fusion of joints separating the maxilla and premaxilla (Fig. 2I,J).

2C-BAC16 mutants also presented sternal defects. The
skeleton forms through migration and medial fusion of two lateral-plate-mesoderm derived elements, which undergo endochondral ossification in regions not contacted by ribs, resulting in the formation of six sternebrae separated by cartilage. It has been suggested that rib heads initially inhibit sternal ossification (Chen, 1952; Braun et al., 1992). In 2C-BAC16 mutants, despite the presence of correct rib number and normal articulation of the sternum with ribs, the fourth and fifth sternebrae were fused, suggestive of an over-riding of the inhibition imposed by ribs (Fig. 2L,M).

These observations show that Fgfr1 signalling resulting from expression of the Pro252Arg mutant receptor selectively affects the development of the skeleton, targets distinct bones and sutures, and affects both membranous and endochondral modes of ossification. The range of defects described here are similar to those reported by other studies of hyperactive Fgfr function in mice (Zhou et al., 2000; Hajihosseini et al., 2001; Wang et al., 2001), and to the cranial and skeletal defects observed in Pfeiffer patients (Muenke et al., 1994; Roscioli et al., 2000). In this respect, it should be noted that some phenotypic features of the 2C-BAC16 model, such as sternal fusions, have not previously been described in Pfeiffer patients. However, it is possible that these defects do occur in Pfeiffer syndrome but have not been previously looked for. It should also be noted that the 2C-BAC16 mice, like the model of Zhou et al. (Zhou et al., 2000), did not show defects in the development of the halluces described in Pfeiffer syndrome.

The phenotype of 4C BAC16 mice: limb and axial skeleton defects

A valuable feature of the BAC model employed in these experiments is the ability to manipulate mutant gene copy number and the consequent expression of mutant transcripts. In particular, it is predicted that a doubling of gene copy number by intercrossing 2C-BAC16 mice would further accentuate signalling via mutant receptors, as it favours the formation of mutant receptor homo-dimers, which are required for mutant gene function in the presence of ligand. 2C-BAC16 mice were intercrossed to produce 4C-BAC16 mice (Fig. 1D), which were recovered in the expected Mendelian ratios.

4C-BAC16 mice presented more severe cranial and sternal phenotypes. The curvature of the maxillary process was accentuated (Fig. 2G,J), resulting in mis-aligned jaws and overgrown or in-growing teeth, particularly the incisors. Fusion accentuated (Fig. 2G,J), resulting in mis-aligned jaws and overgrown or in-growing teeth, particularly the incisors. Fusion of the same animals, L6 often acquired a Sacral (S)1 identity, and C7 acquired a C7 identity, and C7 acquired a Thoracic vertebrae identity (Fig. 3B-D). In one mutant, these transformations were accompanied by fusion of the first and second ribs with an accompanying sternal defect (Fig. 3C). In another, C1 was found to be fused to C2 (not shown). Interestingly though, these defects occurred predominantly in a unilateral fashion, on the left side. In the lower lumbar region of the same animals, L6 often acquired a Sacral (S)1 identity, but this transformation was always bilateral (Fig. 3F). These transformations are similar to those seen in mice carrying a Y766F gain-of-function mutation in Fgfr1 (Partanen et al., 1998), and in those lacking caudal-related genes (Cdx) (Van den Akker et al., 2002).

Defects in axial patterning

The mouse vertebral column is composed of distinct sets of bones that show unit and segmental identity resulting from the expression of a particular set of Homeobox (Hox) genes (reviewed by Gaunt, 2000). A subset of 4C-BAC16s (n=7/15) presented fusion and homeotic transformations of the axial skeleton in a posterior direction. Cervical vertebrae 6 (C6) acquired a C7 identity, and C7 acquired a Thoracic vertebrae 1 (T1) identity (Fig. 3B-D). In one mutant, these transformations were accompanied by fusion of the first and second ribs with an accompanying sternal defect (Fig. 3C). In another, C1 was found to be fused to C2 (not shown). Interestingly though, these defects occurred predominantly in a unilateral fashion, on the left side. In the lower lumbar region of the same animals, L6 often acquired a Sacral (S)1 identity, but this transformation was always bilateral (Fig. 3F). These transformations are similar to those seen in mice carrying a Y766F gain-of-function mutation in Fgfr1 (Partanen et al., 1998), and in those lacking caudal-related genes (Cdx) (Van den Akker et al., 2002).
Preaxial polydactyly in hindlimbs

A majority of 4C-BAC16 mutants exhibited pre-axial polydactyly. These involved the hindlimbs only, occurring either in a unilateral (9/17 right limb only, 2/17 left limb only) or bilateral fashion (Fig. 4; see Table S2 at http://dev.biologists.org/supplemental/). Within each abnormal limb, only digit I (hallux) was affected, and polydactyly was accompanied by overlying soft tissue syndactyly. Skeletal preparations revealed a range of defects, from a broad toe split at the level of phalanx 1 and 2, to complete duplication of digit I bones and their subsequent transformation into two triphalangeal elements (Fig. 4D-J).

The relationship between mutant gene copy number and limb defect was confirmed by breeding 4C-BAC16 mutants with wild-type mice. The resulting 2C mutant offspring reverted to the milder cranial phenotype, and polydactyly was accompanied by overlying soft tissue syndactyly. Skeletal preparations revealed a range of defects, from a broad toe split at the level of phalanx 1 and 2, to complete duplication of digit I bones and their subsequent transformation into two triphalangeal elements (Fig. 4D-J).

Candidate gene analysis of polydactyly in 4C-BAC16 hindlimbs

Much is known about genes that control growth and patterning of the vertebrate limbs in the three primary axes (dorsoventral, anteroposterior and proximodistal) (Martin, 1998; Capdevila and Izpisua Belmonte, 2001). Moreover, targeted mutations in mice or spontaneous mutations in man have implicated a number of genes and pathways in pre-axial polydactyly (see Table S3 at http://dev.biologists.org/supplemental/) (Biesecker, 2002). This provided a list of candidate genes that could mediate the threshold-dependent polydactyly phenotype of 4C-BAC 16 mice. We performed a comprehensive analysis of this candidate gene set, comparing expression patterns in wild-type, 2C-BAC16 and 4C-BAC16 limbs by whole-mount in situ hybridisation. For each gene, the analysis focused on embryonic stages previously shown to correspond with either optimal expression and/or a crucial function.

From the candidate gene set listed in Table S3, the majority, including Shh (Fig. 5A), exhibited a normal expression pattern in both 2C-BAC16 and 4C-BAC16. However, we did detect alterations in the expression patterns of participants in three pathways: the Hox gene pathway, in the form of the d-cluster Homeobox 13 (Hoxd13); the calcium-dependent Wnt pathway, in the form of ligand Wnt5a; and the canonical β-catenin Wnt pathway in the form of Dickkopf (Dkk1).

In wild-type and 2C-BAC16 E11.0 embryos, expression of Hoxd13 was restricted to the posterior two-thirds of the distal hind limb buds, covering domains that generate triphalangeal digits II-V. However, in 4C-BAC16 hind limb buds, this expression extended anteriorly to the region that generates digit I (Fig. 5C). Ectopic expression was maintained at E12.5, when the abnormal hind limbs were easily recognisable by the presence of a larger and broader digit I (Fig. 5E).

At E11.5, Dkk1 is normally expressed throughout the apical ectodermal ridge (AER) and in the hind limbs, strongly in a region that roughly corresponds to parts of the anterior necrotic zone (ANZ) (Fig. 5F,G) (Mukhopadhyay et al., 2001). In eight out of ten 4C-BAC16 hind limbs, the level of Dkk1 expression was reduced in the anterior two-thirds of the AER, and lost in the ANZ (Fig. 5F,H). Cell death in the developing limbs contributes to the final shape of the autopod, and expression patterns and functional data suggests that Dkk1 has a role in inducing cell death (Grotewold and Ruther, 2002). Thus to examine any potential differences in cell death, we labelled E12.5 embryos with Neutral Red dye, a faithful marker of apoptotic cells in developing limbs (Macias et al., 1996; Hajhosseini and Heath, 2002). In normal and 2C hind limbs, extensive cell death was noted in the mesenchyme anterolateral to the digit I condensate. By contrast, and consistent with the differences in Dkk1 expression, we found significantly reduced cell death in the corresponding region of 4C abnormal limbs (Fig. 5I).

Wnt5a has been highlighted as a member of the Wnt family that plays a crucial role in distal limb outgrowth, as well as in...
We thus compared the patterns of 2C- and 4C-mutant limbs, only to discover between wild-type, 2C- and 4C-mutant limbs, only to discover a higher intensity of labelling throughout the distal fore and hind limbs of 4C embryos. More relevant to the described limb expression.

Although we cannot eliminate an increase in the activity of these pathways that does not result in changes in gene regulation properties from those involved in the sternum and threshold signalling dynamics in the development of the skeleton, depending upon the target pathway employed. These findings indicate that a complete understanding of FGF signalling in development requires not only a definition of the connectivity of signalling pathways across different tissues, but also an understanding of signalling dynamics.

Mechanistic properties of signalling thresholds

Threshold signalling may be contrasted with morphogen signalling, in that in the former there is a ‘binary’ response to receptor activation whereas in the latter there is a quantitative relationship between receptor occupancy and response, until saturation of receptors by ligand occurs (Freeman and Gurdon, 2002). In both cases, receptor-mediated activation is controlled by the availability of ligand and the affinity of the ligand for the receptor. In pathways that exhibit threshold properties, a covalent modification (e.g. phosphorylation or ubiquitinylation) is usually linked to a process in which the modification is reversed or inhibited (reviewed by Ferrell, 1999; Saltar and Hofer, 2003; Germain, 2001; Germain and Stefanova, 1999). Thus, the difference between the two types of signalling characteristics is explained by the presence of limiting inhibitory pathways in threshold signalling; in morphogen type systems these inhibitory pathways are absent or compromised (Ortega et al., 2002), and there is therefore a direct relationship between receptor occupancy and signal output. The results reported here therefore show that the development of the axial skeleton and digit I of the hind limb involve Fgfr1-mediated signalling pathways that have different regulatory properties from those involved in the sternum and
cranial sutures. This could include different pathway connectivities and/or the presence or absence of inhibitory regulators.

Morphogen signalling in cranial and sternal defects
Mice harbouring 2C-BAC16 alleles exhibit both endochondral and membranous bone defects, including premature fusion of cranial sutures, the zygapophyseal bones and the sternum. The precocious ossifications and suture fusions reported here are consistent with the proposed role of signalling via Fgf1 in the induction of osteogenic precursor cell terminal differentiation (Colvin et al., 1996; Iseki et al., 1997; Iseki et al., 1999; Rice et al., 2000; Zhou et al., 2000; Eswarakumar et al., 2002; Huang et al., 2003). These sites of action are similar to those observed in the equivalent mutation in man (Muenke et al., 1994; Roscioli et al., 2000), and in a knock-in mouse model reported by Zhou et al. (Zhou et al., 2000). We were, however, also able to show that the severity of these defects increased when the mutant/wild-type expression ratio was doubled, indicating that osteogenic differentiation is accelerated as signalling via Fgf1 increases.

The effects of the mutant Fgf1 gene in these tissues are restricted to specific bones and sutures. In the skull vault, we observed synostosis of metopic (frontal) but not coronal or sagittal sutures. In the skull base, only the joints separating the maxilla from the pre-maxilla were fused. This differential sensitivity of specific sutures to the presence of mutant receptors could represent sites of selective distribution or concentration of FGF ligands, such that Fgf1 signalling is limited by ligand availability in susceptible sutures but is in ligand excess in non-susceptible sutures. Although multiple Fgf ligands are expressed in the developing calvarial sutures (Hajihosseini and Heath, 2002), little is known about the distribution or activity state of the corresponding proteins. However, sites of ligand-limited signalling would be susceptible to the experimental addition of extra ligand. Indeed, Iseki et al. and Greenwald et al. have demonstrated that coronal and metopic sutures undergo premature fusion when exposed to additional Fgf ligand (Iseki et al., 1997; Iseki et al., 1999; Greenwald et al., 2001). Collectively, these findings show that sculpture of the cranium through differential rates of suture fusion in normal development is quantitatively controlled by the availability of Fgf ligand.

Threshold responses in the axial skeleton
4C-BAC16, but not 2C-BAC16, mice exhibit defects in axial patterning whereby distinct vertebrae acquire a more posterior identity. Partanen et al. (Partanen et al., 1998) observed very similar axial transformations, but not polydactyly, by creating a hypermorphic point mutation Y766F in the putative PLCγ/Shb (Cross et al., 2002) docking site in Fgf1. It is significant that this allele does not give rise to polydactyly, indicating that the underlying threshold-dependent pathways in limb and axial skeleton development are distinct.

The vertebral column is derived from the somites, which are generated sequentially and in a rostrocaudal manner from the paraxial mesoderm. Segmentation of the paraxial mesoderm is believed to be regulated by an oscillator mechanism involving the Notch signalling pathway (reviewed by Holley and Takeda, 2002). Fgf8 is expressed in a graded manner caudal to the emerging somites, and ligand overexpression studies have led to the proposal that this corresponds to a wave of Fgf signalling that functions to position segmental boundaries by maintaining the pre-somatic mesodermal cells in an uncommitted state (reviewed by Dubrulle and Pourquie, 2002; Dubrulle et al., 2001; Holley and Takeda, 2002). In this model, the establishment of segment identity occurs when Fgf signalling falls below a predetermined threshold. Our findings support this hypothesis, as it would be predicted that, in the presence of a fixed threshold, the ‘wavefront’ of Fgf1 signalling would extend in a rostral direction in 4C-mutant mice compared with normal counterparts, in turn leading to somites acquiring a more posterior identity (Dubrulle et al., 2001).

Digit I exhibits threshold responses to Fgf1 signalling
Current models of limb development hold that the individual elements (autopod, zupod, stylopod) are specified early. One role of Fgf1 signalling is then to expand each of the specified fields to their relevant final size and shape, by regulating cell proliferation/survival and the expression of genes that pattern each element (Dudley et al., 2002; Sun et al., 2002). Expression patterns and genetic dissection studies suggest that in the early limb bud, the effects of AER-derived Fgfs on the adjacent mesenchyme are transduced by Fgf1 (Peters et al., 1992; Partanen et al., 1998; Eswarakumar et al., 2002). The preaxial ploydactyly in hindlimbs, which occurs when hypermorphic BAC transgene copy number is raised to 4C, shows that the development of digit I, but not digits II-V, is dependent on Fgf1 signalling levels whereby extra digit elements are formed when Fgf1 signalling is elevated above a threshold, defined here by the ratio of mutant to wild-type receptors. By contrast, mice harbouring a hypomorphic Fgf1 allele specifically lack digit I in the hind limb (Partanen et al., 1998). In addition, conditional inactivation of Fgf8 in hind limbs specifically affects digit I development (Lewandoski et al., 2000). We conclude that digit I exhibits a particular dependency on Fgf1 signalling – despite uniform expression of Fgf1 throughout the early limb bud mesenchyme – and that a role of Fgf1 signalling is to regulate the size of the pool of cells destined to give rise to digit I. If Fgf1 signalling is compromised, the digit I precursor pool is reduced leading to the absence of digit I. If, as a result of altered Fgf1 signalling dynamics, the precursor pool is amplified above a threshold level, the outcome is preaxial polydactyly.

How can the dependency of digit I development on Fgf1 signalling levels be explained? First, there could be an asymmetric distribution of Fgf ligands within the AER, such that higher levels in the anterior region corresponding to the presumptive digit I trigger a higher level of mutant receptor activation in the adjacent anterior mesenchyme. However, expression pattern studies to date have shown the converse, with ligands such as Fgf4 being restricted to the posterior AER (Bueno et al., 1996). We have previously shown that Fgf20 is strongly expressed at the anterior and posterior margins of the developing autopod (Hajihosseini and Heath, 2002), but BAC16 mice do not develop digit V polydactyly.

The second, and in our view more likely, explanation is that the development of digit I versus digits II-V is normally governed by two distinct levels of Fgf1 signalling. This supposes that an increase in size of the digit I precursor pool depends upon Fgf1 signalling and is terminated when levels
of Fgfr1 signalling fall below a specified threshold. This threshold mechanism does not operate in the development of digits II-V. In 4C-BAC16 mice, Fgfr1 signalling is elevated and fails to fall below the threshold, resulting in the formation of extra digit elements.

Processes that depend on Fgfr1 signalling in the digit I field could include cell proliferation and/or cell death in the surrounding mesenchyme. Polydactyly in many experimental models is accompanied by a reduction in programmed cell death (Chen and Zhao, 1998; Salas-Vidal et al., 2001). We showed that at E12.5, prior to digit outgrowth, significant cell death does indeed normally occur in the mesenchyme around digit I, and that this is quenched in developing limbs of 4C mutants. We also showed that, in the presence of the hypermorphic 4C allele (but not in the 2C mice), Dkk1 was downregulated close to a region destined to form digit I. Dkk1 expression is associated with regions of programmed cell death in the limb (Grotewold et al., 1999), ectopic expression of Dkk1 in the limb promotes apoptosis (Grotewold and Ruther, 2002) and Dkk1 null mice exhibit polydactyly (Mukhopadhyay et al., 2001). Collectively, this evidence indicates that at least part of the mechanism by which Fgfr1 regulates the digit I precursor field is mediated by a threshold-responsive signal that negatively regulates programmed cell death via target genes such as Dkk1.

The threshold-sensitive relationship between Fgfr signalling and Dkk1 expression/cell death may also hold true for the normal development of digits II-V, as in limb development Dkk1 becomes downregulated in the growing digits and becomes restricted to inter-digital mesenchyme (Grotewold and Ruther, 2002). However, as 4C-BAC16 mice do not develop digit II-V syndactyly, it is likely that this threshold-sensitive control is governed by another Fgf receptor. Fgfr2 would be a good candidate, as gain-of-functions mutations in this gene result in post-axial syndactyly in Apert Syndrome.

4C-BAC16 limbs also exhibit an ectopic anterior shift in the domain of Hoxd13 expression, which is normally restricted to regions that generate the triphalangeal digits II-V. This shift, in addition to the reduced cell death described above, may contribute to the overgrowth in digit I, as mice with loss of Hoxd13 function have significantly shorter digits (Dolle et al., 1993; Bruneau et al., 2001). However, because Hox genes play crucial roles in pattern formation, the most likely consequence of the Hoxd13 shift is the endowment of a triphalangeal identity to the expanded digit I pool.

As the normal expression pattern of genes such as Shh, dHand (Hand2 – Mouse Genome Informatics), Gli3 and several Bmps is unperturbed in 4C-BAC16 limbs, the ectopic Hoxd13 may represent a novel connectivity between Hoxd13 gene expression and elevated Fgfr signalling. This may be mediated by members of the caudal-related homeobox (Cdx) gene family, as Bel-Vialar et al. (Bel-Vialar et al., 2002) have shown that Hox genes exhibit differential sensitivity to Fgf signalling via a Cdx-dependent pathway. In addition, a subset of Cdx1/Cdx2 compound mutant mice develop digit I polydactyly and show posterior hematic transformation of several vertebrae, remarkably similar to those described in this report (Fig. 4) (van den Akker et al., 2002). However, we have been unable to detect expression of these genes in either normal or mutant limbs by WMISH.

### Developmental significance of signalling dynamics

Our studies of the hypermorphic Pro252Arg allele of Fgfr1 have highlighted the importance of signalling dynamics in skeletogenesis. In particular, we have shown that, for Fgfr1, some system responses to ligands are quantitatively related to ligand availability and others exhibit a binary switch in response as signalling passes a threshold value. We have argued that these different categories of response reflect differing designs of downstream signalling pathways. This enables major changes in the developmental process, either evolutionary or pathological, to be effected by changing the ‘values’ of the signalling pathways rather than the connectivity.

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### References


