

Differential regulation of endochondral bone growth and joint development by FGFR1 and FGFR3 tyrosine kinase domains

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SUMMARY

Fibroblast growth factor receptors (FGFR) 1 and 3 have distinct mitogenic activities *in vitro*. In several cultured cell lines, FGFR1 transmits a potent mitogenic signal, whereas FGFR3 has little or no mitogenic activity. However, in other *in vitro* assays the FGFR3 intracellular domain is comparable with that of FGFR1. *In vivo*, FGFR3 negatively regulates chondrocyte proliferation and differentiation, and activating mutations are the molecular etiology of achondroplasia. By contrast, FGFR1 transmits a proliferative signal in various cell types *in vivo*. These observations suggest that inhibition of the proliferating chondrocyte could be a unique property of FGFR3 or, alternatively, a unique property of the proliferating chondrocyte. To test this hypothesis, FGFR1 signaling was activated in the growth plate in cells that normally express FGFR3. Comparison of transgenic mice with an activated FGFR1 signaling pathway with an achondroplasia-like mouse that expresses a similarly activated FGFR3 signaling pathway demonstrated that both transgenes result in

a similar achondroplasia-like dwarfism. These data demonstrate that suppression of mitogenic activity by FGFR signaling is a property that is unique to growth plate chondrocytes. Surprisingly, we observed that in transgenic mice expressing an activated FGFR, some synovial joints failed to develop and were replaced by cartilage. The defects in the digit joints phenocopied the symphalangism that occurs in Apert syndrome and the number of affected joints was dependent on transgene dose. In contrast to the phenotype in the growth plate, the joint phenotype was more severe in transgenic mice with an activated FGFR1 signaling pathway. The failure of joint development resulted from expanded chondrification in the presumptive joint space, suggesting a crucial role for FGF signaling in regulating the transition of condensed mesenchyme to cartilage and in defining the boundary of skeletal elements.

Key words: Fibroblast growth factor, FGF, FGF receptor, Achondroplasia, Mouse

INTRODUCTION

Fibroblast growth factors (FGFs) and FGF receptors (FGFRs) have essential roles in organogenesis and morphogenesis (Szebenyi and Fallon, 1999; Yamaguchi and Rossant, 1995). Autosomal dominant missense mutations in FGFR1-FGFR3 account for a large number of human skeletal dysplasia and craniosynostosis syndromes (Burke et al., 1998; Naski and Ornitz, 1998; Wilke et al., 1997). Biochemical and genetic studies indicate that most of the point mutations in FGFRs result in increased or ectopic FGFR signaling (Naski et al., 1996; Neilson and Friesel, 1996; Webster and Donoghue, 1996). Molecular mechanisms by which mutations in FGFRs activate signaling include constitutive ligand-independent receptor dimerization (Galvin et al., 1996; Naski et al., 1996; Robertson et al., 1998), increased ligand-binding affinity (Anderson et al., 1998), altered ligand-binding specificity (Yu et al., 2000) and decreased ligand-mediated receptor downregulation (Monsonogo-Ornan et al., 2000).

Gain-of-function mutations in FGFR3 inhibit endochondral

bone growth and cause the diseases hypochondroplasia, achondroplasia and thanatophoric dysplasia. By contrast, mutations in FGFR2, and a single mutation in FGFR1, are associated with the craniosynostosis syndromes, some of which also include phenotypes affecting the appendicular skeleton (Burke et al., 1998; Naski and Ornitz, 1998; Wilke et al., 1997). The phenotype of each syndrome correlates with a specific FGFR mutation, and with the spatial expression pattern of FGFRs in mesenchymal condensations and in developing endochondral and membranous bone (Delezoide et al., 1998; Iseki et al., 1999; Johnson et al., 2000; Orr-Urtreger et al., 1991; Peters et al., 1992).

In growing long bones with established growth plates, FGFR3 is highly expressed in proliferating chondrocytes and acts to inhibit proliferation (Colvin et al., 1996; Deng et al., 1996; Naski et al., 1998; Naski and Ornitz, 1998; Naski et al., 1996; Peters et al., 1992; Webster and Donoghue, 1996; Webster and Donoghue, 1997b). This activity of FGFR3 is remarkable considering the classical view that FGFs and their receptors transmit mitogenic signals. This raises the question

of whether the inhibition of chondrocyte proliferation is a unique property of FGFR3 or a unique property of the chondrocyte.

In vitro, activated FGFR3 inhibits proliferation of several cell types. In 293T cells, constitutively active FGFR3 (containing the activation loop mutation K650E) specifically activated the transcription factor STAT1, which upregulates p21 expression, a known cell cycle inhibitor (Su et al., 1997). This observation was supported by the study of *Stat1*^{-/-} bone explants, in which treatment with FGF was unable to inhibit longitudinal growth (Sahni et al., 1999). In CFK2 chondrocytes, FGFR3 (containing the weakly activating transmembrane domain mutation G380R), inhibited cell growth (Henderson et al., 2000). In contrast to these data, intracellular domains of FGFR1, FGFR3 or FGFR4 containing the constitutive activation loop mutation K650E and a plasma membrane targeting myristylation signal, all induced a transformed phenotype in NIH3T3 cells (Hart et al., 2000; Webster and Donoghue, 1997a). Furthermore, chromosomal translocations involving FGFR3 and constitutive activating mutations have been implicated as the etiological agent of some bladder carcinomas and some forms of myeloma (Cappellen et al., 1999; Chesi et al., 1997; Plowright et al., 2000; Richelda et al., 1997). These data demonstrate that constitutively activated FGFR3 can be mitogenic for some cell types.

In contrast to *Fgfr3*, which is expressed in proliferating chondrocytes, *Fgfr1* is expressed in the adjacent hypertrophic chondrocytes and in articular chondrocytes. *Fgfr1* and *Fgfr2* are expressed in the perichondrium (Delezoide et al., 1998; Orr-Urtreger et al., 1991; Peters et al., 1993; Peters et al., 1992). The function of FGFR1 and FGFR2 in endochondral bone growth is not known; however, the non-overlapping expression patterns of FGFR1-FGFR3 suggest that these receptors have unique functions, mediated by differences in their ligand-binding specificity and/or downstream signaling.

The FGFR intracellular region contains a juxta-membrane domain, a bipartite tyrosine kinase domain and a kinase insert sequence, and is responsible for signal transduction. The intracellular regions of FGFR1 and FGFR3 share 73% amino acid sequence identity. Several studies have demonstrated differences in FGFR signaling potency in a variety of in vitro assays. In BaF3 cells, FGFR1 and FGFR2 elicit a strong mitogenic response whereas FGFR3 and FGFR4 fail to maintain cell proliferation, even in the presence of saturating ligand concentrations (Naski et al., 1996; Ornitz et al., 1996; Wang et al., 1994). Similarly, in PC12 cells, FGFR1 elicits a potent neurite outgrowth response, while FGFR3 shows less activity (Lin et al., 1996; Raffioni et al., 1999). However, when the intracellular domain of FGFR3 or FGFR4 is replaced with the intracellular domain of FGFR1, activity is restored in both cases (Naski et al., 1996; Wang et al., 1994). This suggests that sequence differences between intracellular regions of FGFR1 and FGFR3/FGFR4 have different signaling abilities. Additionally, mapping studies have localized a region in the juxta-transmembrane domain of FGFR1 that is required for neurite outgrowth in PC12 cells but not for mitogenesis in BaF3 cells (Lin et al., 1998). Thus, biochemical evidence supports the hypothesis that sequence differences between the FGFR1 and FGFR3 intracellular domains account for differences in signal transduction.

To test the hypothesis that inhibition of proliferating chondrocytes is a unique property of FGFR3 signaling, the regulatory elements of the gene for type II collagen were used to over express either a weakly activated FGFR3 (G380R) (Naski et al., 1998) or a chimeric FGFR (FGFR31, containing the extracellular and transmembrane domains of FGFR3 (G380R) and the intracellular domain of FGFR1) in the growth plate. Comparison of the phenotypes of mice expressing these transgenes demonstrated that activation of FGFR1 signaling pathways mimicked the effect of FGFR3 in proliferating chondrocytes. These data support the alternative hypothesis that FGFR1 and FGFR3 intracellular domains have similar signaling properties in proliferating chondrocytes and that unique properties of proliferating chondrocytes predisposes to growth arrest in response to an FGF signal. Interestingly, we also observed that over-activation of FGFR signaling in cartilage tissue prevented proximal joint formation and distally caused a reduction in the number of phalangeal elements. This observation suggests a role for FGF signaling in the transition from condensed mesenchyme into cartilage tissue and in defining the boundaries of skeletal elements.

MATERIALS AND METHODS

Transgenic mice

The ColIII-FGFR31^{ach} transgene was constructed as described by Naski et al. (Naski et al., 1998). However, the FGFR3^{ach} cDNA was replaced with a chimeric FGFR31 cDNA containing the extracellular and transmembrane domain of FGFR3 (with or without the G380R transmembrane point mutation), and the intracellular domain from FGFR1 (Naski et al., 1996). The ColIII-FGFR31^{ach} cDNA was excised from vector sequence with *AflIII* and *NotI*. The linear DNA fragment was purified by agarose gel electrophoresis, isolated by electro-elution and injected into fertilized mouse oocytes.

Genotyping

Transgenic mice were generated and maintained in an FVB/N genetic background. Heterozygous mice were identified by PCR amplification of hGH cDNA in the 3' untranslated region of the transgene as described previously (Naski et al., 1998). Homozygous mice were identified by DNA blotting of *BglII* restricted tail DNA hybridized to a *Fgfr3*-specific probe that recognizes both transgene and endogenous FGFR3 fragments. Homozygous mice were determined by the intensity ratio of the endogenous FGFR3 fragments to the transgene band.

In situ hybridization

The probes detected hGH exon V (T. Simon, St Louis, MO), type II collagen (Y. Yamada, Rockville, MD, USA) and GDF5 (D. Kingsley, Palo Alto, CA). In situ hybridization was carried out as described previously (Naski et al., 1998).

Histology

Tissues were fixed in 4% paraformaldehyde or 10% formalin. The skeletal tissues were decalcified in 14% EDTA (pH 8.0), embedded in paraffin and stained with Hematoxylin and Eosin.

BrdU labeling

Mice received an intraperitoneal injection of bromodeoxyuridine (BrdU) at a dose of 100µg/g body weight and were sacrificed after 1 hour. Tissues were processed as described above and immunostained for BrdU as described previously (Naski et al., 1998).

Skeletal preparation and bone morphometry

Skeletons were prepared as described previously (Colvin et al., 1996). Bone length was determined using Foster-Findley image analysis software.

RESULTS

Expression of FGFR3 (G380R) and FGFR31 (G380R) in growth plate chondrocytes

Transgenic mice expressing a type II collagen promoter/enhancer-*Fgfr3* cDNA, which contains the weakly activating G380R (achondroplasia) mutation (ColII-FGFR3^{ach}), develop a short limb dwarfism phenotype similar to that seen in human achondroplasia (Naski et al., 1998; Wang et al., 1999). Growth plate chondrocytes in these mice show decreased proliferation and delayed differentiation. By contrast, in mice that lack *Fgfr3*, chondrocyte proliferation is increased and differentiation is accelerated (Colvin et al., 1996; Deng et al., 1996). These data demonstrate that *Fgfr3*, which is expressed specifically in proliferating chondrocytes, negatively regulates their growth. The hypothesis that FGFR1 and FGFR3 have unique signaling activities in chondrocytes is based on this paradoxical activity of FGFR3, on several in vitro assay systems in which FGFR3 signals poorly compare with FGFR1, and because FGFR1 and FGFR3 have adjacent non-overlapping expression patterns in the growth plate. To test this hypothesis, we constructed a chimeric receptor molecule (FGFR31^{ach}) in which the intracellular domain of FGFR3^{ach} was replaced with the intracellular domain of FGFR1 (Fig. 1A). This type of chimeric construct was chosen to maintain the ligand binding and receptor activation properties constant, and to only vary the intracellular signaling domain.

Two transgenic mouse lines (line 1 and 2) that transmit the ColII-FGFR31^{ach} transgene, were established and examined for transgene expression pattern and phenotype. The relative expression pattern of the transgene in both ColII-FGFR31^{ach} lines was compared with that of ColII-FGFR3^{ach} mice by in situ hybridization on age-matched limb tissue. The spatial pattern of transgene expression was similar in all three lines and included resting and proliferating chondrocytes and excluded hypertrophic chondrocytes (Fig. 1B and data not shown). The level of transgene expression in ColII-FGFR31^{ach} line 2 and ColII-FGFR3^{ach} mice was similar, and the level of expression in ColII-FGFR31^{ach} line 1 mice was approximately 50% lower (Fig. 1B). One line of wild-type ColII-FGFR31 transgenic mice was also generated as a control. These mice expressed the transgene in a similar pattern and at a similar level, and showed no apparent phenotype (data not shown).

FGFR1 signaling negatively regulates long bone growth

Both ColII-FGFR31^{ach} transgenic lines exhibited a dwarfism phenotype with characteristics that are strikingly similar to ColII-FGFR3^{ach} transgenic mice. Both types of mice display shortened long bones and a domed-shaped skull, probably owing to craniofacial hypoplasia (Fig. 1C). The severity of the observed dwarfism phenotype correlated with transgene expression level. The body weight of line 1 mice was 85% of littermate controls and line 2 mice was 60-70% of controls (Table 1A). Morphometric comparison of ColII-FGFR31^{ach}

Table 1. Morphometric data

(A) Body weight				
Genotype	<i>n</i>	Mean weight (g)±s.d.*	Body weight (% of control)	<i>P</i> ‡
Wild type	4	18.3±1.1		
FGFR31 ^{ach} -1	4	15.5±0.5	85	<0.01
Wild type	5	18.4±0.7		
FGFR31 ^{ach} -2	4	11.8±1.2	64.3	<0.01
Wild type	4	18.0±1.3		
FGFR3 ^{ach}	4	11.1±4.9	61.7	<0.05

(B) Bone length

Genotype	<i>n</i>	Mean tibia length (mm)±s.d.§	Tibia length (% of control)	<i>P</i> ‡
Wild type	5	15.1±0.3		
FGFR31 ^{ach} -2	4	13.1±0.3	86.7	<0.05
Wild type	4	16.1±0.1		
FGFR3 ^{ach}	6	14.1±0.6	87.8	<0.05

*Body weight was measured at postnatal day 24-26 in heterozygous transgenic mice and in wild-type littermates.

‡*P* was calculated using Stat View Software.

§Tibia length was measured along the midline of the bone between the epiphyseal growth plates using Foster-Findley PC Image Software. Heterozygous FGFR31^{ach} line 2 mice were 24-25 days old and FGFR3^{ach} mice were 24-26 days old. Wild-type littermates were used as controls.

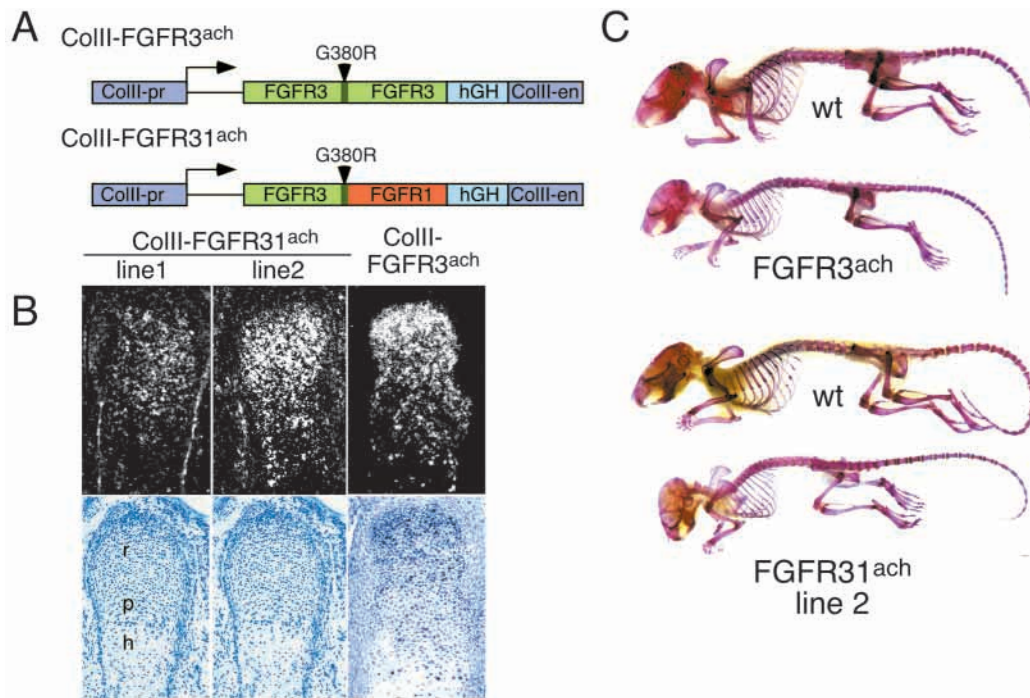
line 2 mice and ColII-FGFR3^{ach} mice demonstrated a similar degree of dwarfism (Table 1B). In both lines of mice, the length of the tibia was reduced by 13-14% at postnatal day 24-26. Both body weight comparisons and tibial length comparisons showed no statistically significant differences between ColII-FGFR31^{ach} line 2 mice and ColII-FGFR3^{ach} mice. Similarly, histological examination of the growth plate revealed strikingly similar features in both lines of mice (Fig. 2A-C). Both the hypertrophic and proliferating zones were significantly decreased in length compared with wild-type littermates, and the formation of secondary ossification centers was delayed in both transgenic lines. Consistent with histological observations and previous data, cell proliferation in the proliferating zone chondrocytes was similarly decreased in both ColII-FGFR31^{ach} line 2 mice and ColII-FGFR3^{ach} mice (Fig. 2D-G).

ColII-FGFR31^{ach} and ColII-FGFR3^{ach} mice develop a segmentation defect in the sternum and fusion of phalangeal joints

Bony segments are joined at synovial and synarthrotic joints. Synovial joints contain an articular surface, which is frequently subjected to movement. The synarthrotic, or non-synovial joints allow little movement. The development of a joint requires the early specification of the presumptive joint position within the condensed mesenchymal anlage (Haines, 1947). It is not clear how this specification occurs. *Wnt14* and *Gdf5* are the two earliest markers that are known to be expressed specifically in the presumptive joint positions, suggesting crucial roles in joint development (Hartmann and Tabin, 2001; Storm et al., 1994). GDF5 null mice fail to form approximately 30% of their synovial joints, indicating that GDF5 is essential for the development of some joints. Other BMP family members, such as BMP5, may also participate in joint patterning (Storm and Kingsley, 1996). In joint development, cells in the specified presumptive joint space do

Fig. 1. Generation of transgenic mice expressing the FGFR3^{1ach} transgene in chondrocytes.

(A) Map of the ColII-FGFR3^{1ach} and ColII-FGFR3^{3ach} transgene. The transgene expression vector contains the type II collagen promoter (ColII-pr), the β -globin splice donor and acceptor (black line), the FGFR cDNA including the G380R transmembrane domain mutation, the hGH gene including polyadenylation sequences, and the enhancer sequences of the rat type II collagen gene (ColII-en). FGFR3 sequences are in green. FGFR1 intracellular domain sequences are in red. (B) Comparison of transgene expression patterns and levels by in situ hybridization using an antisense hGH riboprobe on E18.5 tissues. The transgene expression domains are consistent between ColII-FGFR3^{1ach} and ColII-FGFR3^{3ach} mice, which include resting (r) and proliferating (p) chondrocytes. Expression is excluded from hypertrophic (h) chondrocytes. (C) Alizarin Red-stained skeletons of 1 month old transgenic mice and wild-type littermates. The ColII-FGFR3^{1ach} line 2 transgenic mice and ColII-FGFR3^{3ach} transgenic mice show a similar degree of dwarfism.



not complete chondrification, while the two adjacent regions form chondrocyte islands. Recent studies in chick embryos show that WNT14 may function as the signal repressing the chondrification of the cells located in the joint space. Later in development, the joint space can be identified as a three-layered interzone consisting of centrally localized flattened non-chondrogenic cells surrounded by chondrogenic islands. Owing to changes in the extracellular matrix and other factors such as fetal movement, joint cavitation initiates at the periphery and spreads centrally (Francis-West et al., 1999). In addition to defects in synovial joint formation, mice deficient in either GDF5 or BMP5 develop defects in sternal segmentation with the joint between sternbrae 3 and 4 most frequently affected (Storm and Kingsley, 1996).

Both ColII-FGFR3^{1ach} and ColII-FGFR3^{3ach} heterozygous transgenic mice developed defects in synovial and non-synovial joints. The middle phalangeal joint of the second and third digits in ColII-FGFR3^{1ach} line 2 and the second digit of ColII-FGFR3^{3ach} mice failed to develop. Both forefeet and hindfeet were affected with 100% penetrance ($n=15$). The occurrence of this phenotype in both FGFR3^{3ach} and FGFR3^{1ach} transgenic lines indicated that this was not a consequence of insertional mutagenesis. Histological sections revealed that a cartilaginous remnant is present in the presumptive interphalangeal joint position with two opposing growth plates (Fig. 3B,D) suggesting that the initial specification of the

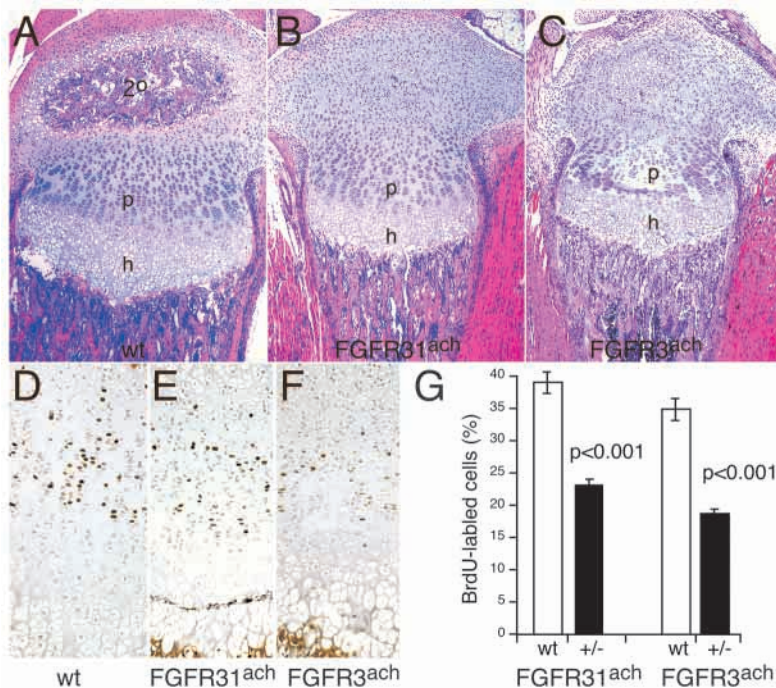


Fig. 2. Histology and cell proliferation in the proximal tibial epiphyseal growth plate at postnatal day 10. (A-C) Hematoxylin and Eosin stained sections of wild-type mice (A), ColII-FGFR3^{1ach} transgenic mice (B) and ColII-FGFR3^{3ach} transgenic mice (C). (D-F) Immunohistochemical detection of BrdU-labeled growth plate chondrocytes of wild-type (D), ColII-FGFR3^{1ach} transgenic mice (E) and ColII-FGFR3^{3ach} transgenic mice (F). (G) Quantification of the percentage of BrdU-positive cells in the epiphyseal growth plate of the proximal tibia ($n=4-5$).

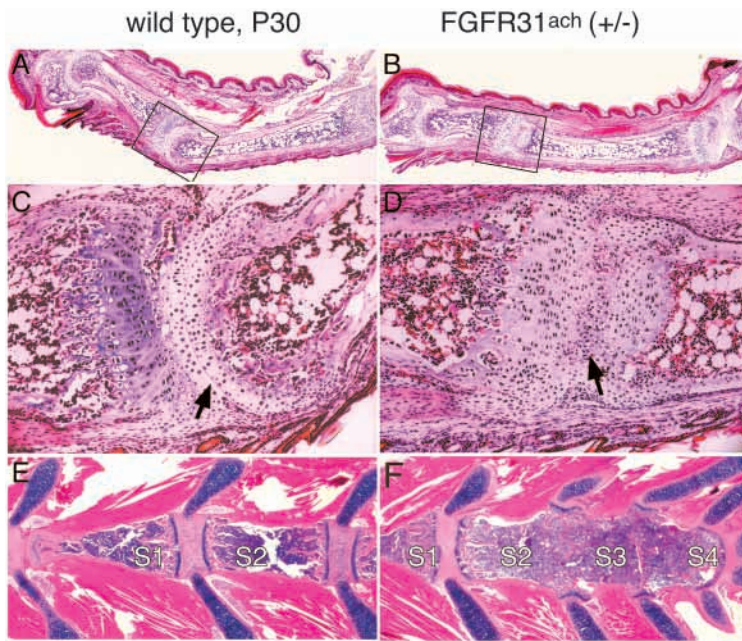


Fig. 3. Synovial and non-synovial joint fusion in heterozygous ColIII-FGFR31^{ach} transgenic mice at postnatal day 30. (A,B) Section through the middle phalangeal joint of the front first digit of wild-type (A) and ColIII-FGFR31^{ach} transgenic mice (B). The boxed region is shown at a higher magnification in C,D. (E,F) Section through the sternbrae of wild-type mice (E) and ColIII-FGFR31^{ach} transgenic mice (F). The joints between S2, S3 and S4 in transgenic tissue are completely replaced by bone marrow.

presumptive joint has occurred normally. Additionally, the non-synovial joints of the sternum were also affected. The wild-type sternum is segmented into four sternbrae, and in both the FGFR3^{ach} and FGFR31^{ach} transgenic lines, the joints between S2, S3 and S4 failed to form (Fig. 3E-F, and data not shown). Movement is known to play a crucial role in joint cavitation. Because non-synovial joints that are rarely subjected to movement were also affected in FGFR3^{ach} and FGFR31^{ach} transgenic mice, the defect in joint development is unlikely to result from lack of movement.

Increased transgene expression results in widespread joint fusion

To further investigate the role of FGFR signaling in joint development, we increased the gene dose and transgene expression by generating homozygous transgenic mice. Homozygous ColIII-FGFR31^{ach} line 2 mice were the most severely affected and died within 1-2 hours of birth. Examination of skeletal histology revealed that additional joints were fused in homozygous animals, suggesting a dose-dependent response to transgene expression. The joints affected in homozygous ColIII-FGFR31^{ach} line 2 mice included the knee, elbow, hip (with incomplete penetrance) and all phalangeal joints (Figs 4, 5 and data not shown). Shoulder

joints appeared to develop normally. In homozygous ColIII-FGFR31^{ach} line 1 mice, in which transgene expression is lower, the elbow and phalangeal joints (Fig. 5) were fused, but the knee joints developed normally. Because partial joint fusion was never observed, these data suggest that successful joint development responds to a threshold level of FGF signaling.

The joint fusion phenotype of homozygous ColIII-FGFR3^{ach} mice was milder than homozygous ColIII-FGFR31^{ach} line 2 mice (Table 2) even though transgene expression levels in these two lines of mice are comparable. In homozygous ColIII-FGFR3^{ach} mice, the elbow joints were also affected but the knee joints developed normally. Phalangeal joints were similarly affected in homozygous and heterozygous mice. This suggests that joint development may be more potently affected by FGFR1 kinase domain signals than FGFR3 kinase domain signals. This may be due to quantitative differences in FGFR signaling, similar to that observed in vitro (Lin et al., 1998). Recently, it has been shown that the G380R mutation caused a delay in ligand-mediated internalization of FGFR3, which could contribute to elevated signaling from the mutant receptor (Monsonego-Ornan et al., 2000). It is therefore possible that quantitative differences in joint development could be a consequence of increased expression of the FGFR31^{ach} protein compared with

Table 2. Joint fusion in heterozygous and homozygous transgenic mice

Transgene dosage	FGFR31 ^{ach}				FGFR3 ^{ach}	
	Line 1		Line 2		+/-	+/+
	+/-	+/+	+/-	+/+		
Transgene expression level	+	++	++	++++	++	++++
Digit joint fusion	-	++++	+	++++	+	+
Knee joint fusion	-	-	-	+	-	-
Elbow joint fusion	-	+	-	+	-	+
Sternebrae fusion	-	ne	+	ne	+	ne

ne, not examined.

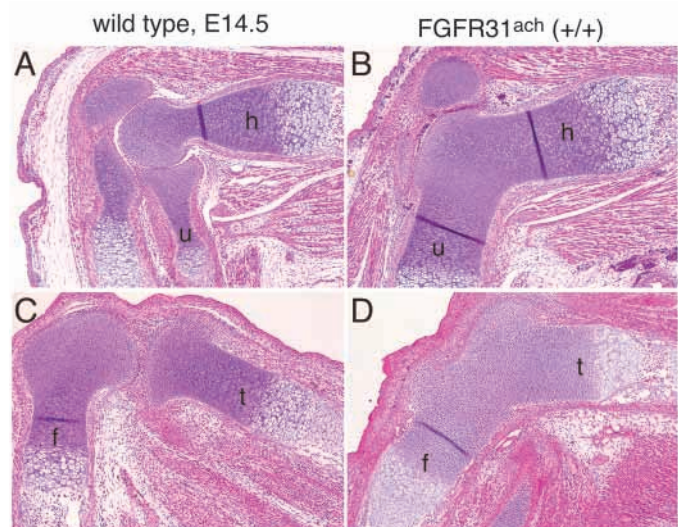


Fig. 4. Elbow and knee joint fusion in homozygous ColIII-FGFR31^{ach} transgenic mice (line 2) at E15.5. (A,B) Elbow joint of wild-type (A) and ColIII-FGFR31^{ach} transgenic line 2 (B). (C,D) Knee joints of wild-type (C) and ColIII-FGFR31^{ach} transgenic line 2 (D). f, femur; h, humerus; t, tibia; u, ulna.

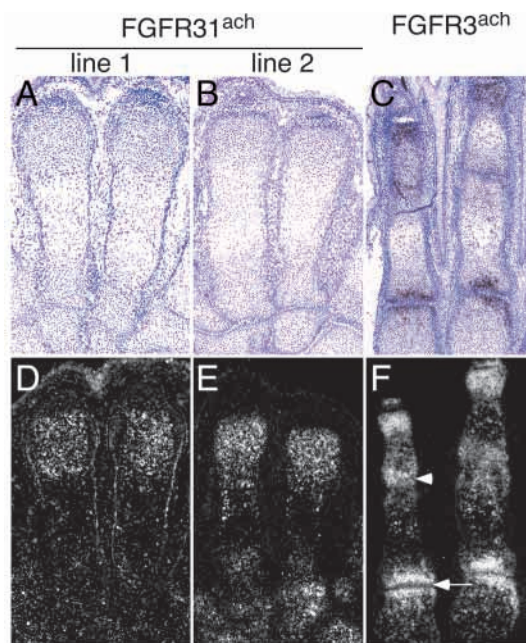


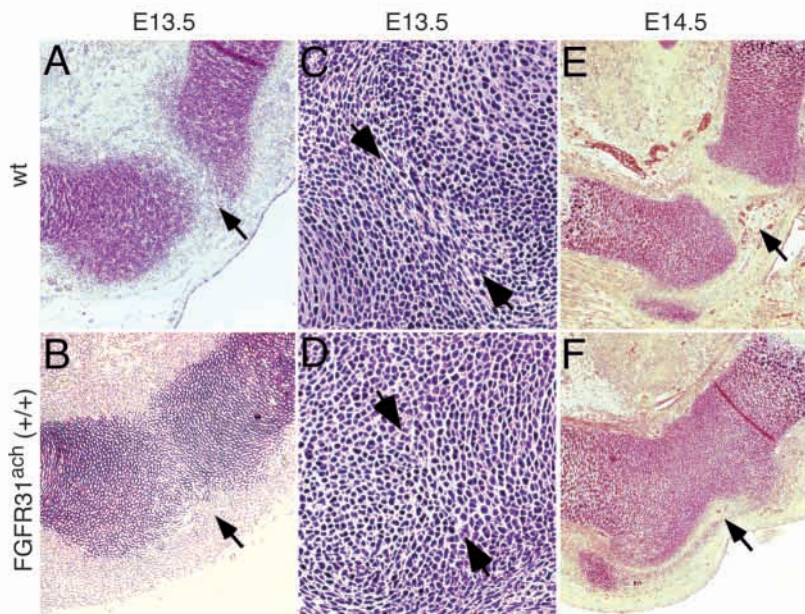
Fig. 5. Loss of phalangeal joints in homozygous ColIII-FGFR3^{ach} transgenic mice at E17.5. (A-C) Hematoxylin stained forelimb digits 2 and 3. (D-F) In situ hybridization showing transgene (hGH) expression. In both ColIII-FGFR3^{ach} transgenic line 1 (A,D) and line 2 (B,E), the joints in the second and third digits of the forefeet are completely lost and replaced with a single cartilaginous element. In homozygous ColIII-FGFR3^{ach} transgenic mice (C,F), the proximal phalangeal joints develop normally (indicated by an arrow), but the distal phalangeal joint is fused (indicated by an arrowhead).

the FGFR3^{ach} protein. However, these quantitative differences are only evident at higher receptor expression levels because heterozygous ColIII-FGFR3^{ach} mice and ColIII-FGFR3^{ach} line 2 mice do not differ in the severity of joint fusion.

Joint fusion results from expanded chondrification and correlates with decreased *Gdf5* expression

Histological analysis of phalangeal and limb bones revealed that a cartilaginous remnant and two opposing growth plates remained in the

Fig. 6. Type II collagen expression and cell morphology in the knee joint of homozygous ColIII-FGFR3^{ach} mice at E13.5 and E14.5. (A) At E13.5, the presumptive joint position (indicated by an arrow) is well defined by a collagen II-negative zone in wild-type tissue. (B) The presumptive joint space in tissue from homozygous ColIII-FGFR3^{ach} transgenic mice has diffuse type II collagen expression, indicating the initiation of chondrification of the joint interzone. (C,D) Cell morphology in the developing joint. (C) Wild-type E13.5 joint tissue showing elongated mesenchymal cell shape. (D) Homozygous ColIII-FGFR3^{ach} line 2 transgenic tissue showing a rounded cell morphology. The presumptive joint space is located between the arrows. (E) The type II collagen-free region (arrow) in the developing knee joint in the wild-type animal is well established by E14.5. (F) The knee joint (arrow) is completely replaced by type II collagen-expressing cells in homozygous ColIII-FGFR3^{ach} line 2 tissue.



presumptive joint region (Figs 3, 4). This suggests normal initial specification of presumptive joint position. The failure of joint formation must therefore result from a failure to maintain the joint interzone or a failure in joint cavitation. The earliest histological evidence of knee joint cavitation appears around E15 in the mouse. In situ hybridization for type II collagen mRNA at E13.5 and E14.5 (prior to cavitation) was used to identify the region occupied by chondrocytes. In sections from wild-type limbs at E13.5 (Fig. 6A), the boundary between type II collagen-positive and -negative zones was clearly established. In limb tissue from homozygous ColIII-FGFR3^{ach} line 2 transgenic animals, the boundary between type II collagen-positive and -negative regions was indistinct (Fig. 6B), suggesting expanded chondrification of mesenchymal cells in the presumptive joint space. Furthermore, the morphology of cells in the joint space in homozygous transgenic mice adopted a rounded shape, a characteristic of chondrocytes (Fig. 6D). By contrast, cells occupying the presumptive joint space in wild-type tissue adopted an elongated shape (Fig. 6C). Corresponding with these morphological changes, cell proliferation in the joint zone of homozygous ColIII-FGFR3^{ach} mice, determined by BrdU labeling, was decreased compared with that of wild-type mice at E13.5 (Fig. 7). In contrast to the decreased proliferation in the joint space at E13.5, proliferation in the growth plate was comparable between wild-type and homozygous ColIII-FGFR3^{ach} mice. This suggests that the joint fusion is not secondary to the decreased proliferation of chondrocytes in the growth plates. Cell death, determined by TUNEL labeling, was detected in very few cells and there were no apparent differences between wild-type and homozygous ColIII-FGFR3^{ach} joint zones (data not shown).

Cavitation of the joint between the tibia and femur initiates after E14.5. However, by E14.5, the boundary between the future joint and neighboring cartilage is clearly demarcated into type II collagen-positive and -negative zones in wild-type tissue (Fig. 6E). By contrast, in tissue from homozygous ColIII-FGFR3^{ach} line 2 transgenic mice, the joint space was obliterated and replaced by a continuous zone of type II

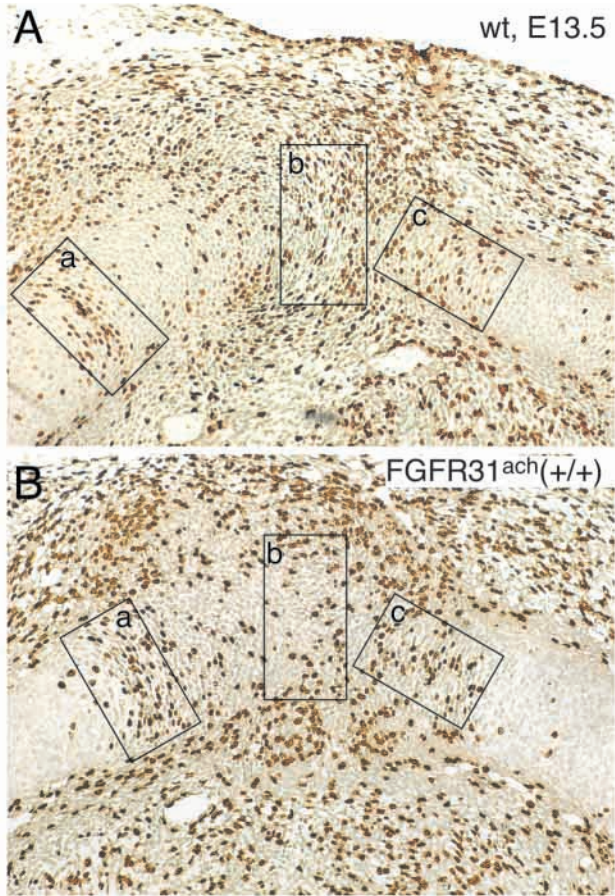


Fig. 7. Pattern of cell proliferation in the knee joint at E13.5 in wild-type and homozygous ColII-FGFR31^{ach} line 2 mice. (A) Wild-type and (B) homozygous transgenic tissue stained for BrdU incorporation. Box a and Box c outline the growth plates of the tibia and femur, respectively. Box b outlines the region of the presumptive joint. The number of proliferating cells in the growth plates of the tibia and femur is comparable between wild-type and homozygous transgenic tissue, whereas there is a significant decrease in the number of proliferating cells in the presumptive joint space in transgenic tissue compared with wild-type tissue.

joint boundary can still be identified by a region of decreased type II collagen expression (Fig. 6B). Notably, *Gdf5* expression was completely lost in the joint interzone and dramatically reduced in the perichondrium. Only a trace amount of GDF5 expression was observed at the dorsal edge ($n=3$ out of 3). This observation suggests that expanded chondrification initiates centrally and progresses laterally. Examination of transgene expression in heterozygous and homozygous limb tissue (Fig. 8A,B, inset) demonstrated that ColII-FGFR31^{ach} expression was excluded from the presumptive joint space and was complementary to that of *Gdf5*. In homozygous ColII-FGFR31^{ach} tissue, transgene expression expanded diffusely into the presumptive joint space.

DISCUSSION

Both in vitro and in vivo studies demonstrate that FGFR3 inhibits chondrocyte proliferation. Despite the high homology in their intracellular domains, FGFR1 and FGFR3 have very different signaling potencies in a variety of cell types in vitro. FGFR1 elicits a strong response in BaF3 and PC12 cells, whereas FGFR3 does not. In this study, we show that in growth plate chondrocytes, activating FGFR1 or FGFR3 signaling pathways had comparable negative proliferative effects. In

collagen-expressing cells (Fig. 6F). These data suggest that the failure of joint development occurred before the cavitation stage and resulted from the expansion of type II collagen-expressing chondrocytes into the presumptive joint space.

Gdf5 is one of the earliest known markers of the presumptive joint. *Gdf5* is also expressed in perichondrium. *Gdf5* expression was therefore examined in ColII-FGFR31^{ach} line 2 transgenic limb tissue at E13.5 (Fig. 8). In wild-type tissue, type II collagen-negative regions expressed high levels of *Gdf5*. This is consistent with other studies, which localized *Gdf5* expression in the presumptive joint space as early as E11.5 (Storm and Kingsley, 1999). In tissue from homozygous ColII-FGFR31^{ach} line 2 transgenic limb tissue, the

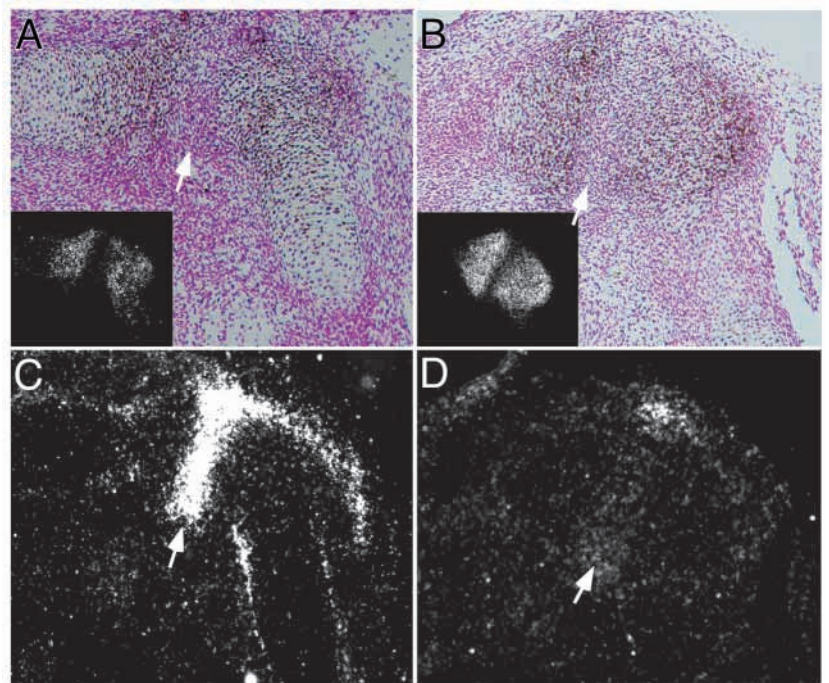


Fig. 8. *Gdf5* expression in the joint space (arrows) of heterozygous (A,C) and homozygous (B,D) ColII-FGFR31^{ach} line 2 mice at E13.5. (A,B) Hematoxylin and Eosin stained knee joint tissue. Inserts in (A,B) are the dark-field images showing transgene (hGH) expression. (C,D) Dark-field images showing *Gdf5* expression. (C) In heterozygous tissue, *Gdf5* is robustly expressed in the joint space and is complementary to the transgene expression domain. (D) In homozygous tissue, *Gdf5* expression is dramatically reduced, even though ColII expression identifies the presumptive joint space. This indicates that loss of *Gdf5* expression precedes complete joint fusion.

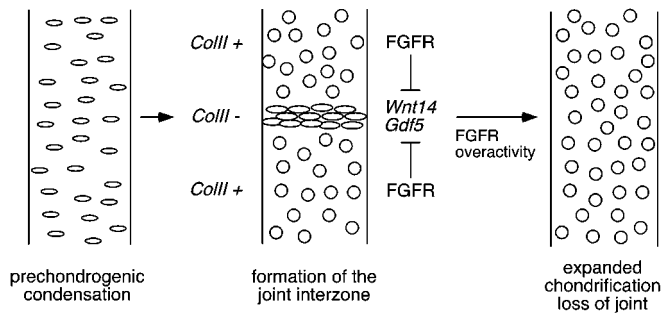


Fig. 9. Model showing FGF regulation of joint development. The formation of the joint interzone is marked by the appearance of *Wnt14* and *Gdf5* expression, and decreased type II collagen expression. Joint interzone cells also have an elongated cell morphology. Adjacent cartilage elements express higher levels of type II collagen and express the mutant FGFR. At later stages in ColIII-FGFR31^{ach} and ColIII-FGFR3^{ach} mice, chondrification expands into the joint interzone owing to over activation of the FGFR signaling pathway in adjacent chondrocytes, resulting in a failure of joint formation.

addition, overactivation of FGFR signaling in developing cartilage caused a failure of joint cavitation as a result of expanded chondrification. In this cellular context, FGFR1 is more potent than FGFR3.

Inhibitory effect of FGF receptor signaling on cell proliferation is a unique property of proliferating chondrocyte

Activation of FGFRs results in auto-phosphorylation of tyrosine residues in the intracellular domain, which triggers mitogen-activated protein kinase, phospholipase C γ and PI-3 kinase signaling cascades. The cellular response to FGFR activation is complex and sometimes paradoxical, and includes proliferation, anti-proliferation, differentiation, migration or apoptosis. The extent to which this wide spectrum of responses results from differences between the four FGFR signaling domains was not known. The results of this study suggest that cellular context, and to a lesser extent differences in signaling capacity, are crucial determinants of the cellular response to FGF. This is consistent with increasing evidence that different FGFRs can activate largely overlapping downstream signaling pathways with varying potency (Raffioni et al., 1999). In chondrocytes, STAT1 has been implicated as a key signaling molecule that mediates the anti-proliferative activity of FGFR3 (Sahni et al., 1999). Interestingly, it has recently been demonstrated that activation of FGFR1 or FGFR3 can activate STAT family members in NIH3T3 and PC12 cells (Hart et al., 2000). This observation provides a possible molecular basis for the inhibitory effect of FGFR1 on chondrocyte proliferation.

FGF receptors function in joint development

Expression studies and human genetic diseases suggest that FGFR signaling is required for normal joint development. FGFR2 is expressed early in prechondrogenic condensations and later in the perichondrium, periosteum and periarticular cartilage (Delezoide et al., 1998). In Apert syndrome, which results from mutations in *Fgfr2*, the proximal interphalangeal joints are absent at birth, and there is a gradual loss of distal phalangeal joints with age (Green, 1982; Holten et al., 1997).

Cases of lateral cartilaginous fusion of the digits have also been documented (Cohen and Kreiborg, 1995). We show that both ColIII-FGFR31^{ach} and ColIII-FGFR3^{ach} transgenic mice develop phalangeal joint fusion that is suggestive of the phenotype seen in Apert syndrome. This probably results from transgene expression in resting chondrocytes near the presumptive articular surface. This region of type II collagen expression overlaps the endogenous *Fgfr2* expression domain. In human embryos, FGFR2 is intensely expressed in the developing elbow joints and the adjacent cartilage tissues (Delezoide et al., 1998). Thus, the function of the ColIII-FGFR transgenes in joint development may be similar to that of endogenous *Fgfr2* in vivo. This supports the hypothesis that the cellular context is the predominant factor in determining the effect of FGFR signaling. Furthermore, these data suggest that morphogenesis of joints at different physical positions responds to a graded threshold of FGFR activation. Phalangeal joints are affected by lower levels of FGFR signaling compared with knee and elbow joints. This may explain why joint fusion in non-phalangeal joints has not been observed in individuals with activating mutations in FGFR2.

Interestingly, a mouse model that resembles Apert/Pfeiffer syndromes also develops a sternal joint fusion phenotype (Hajihosseini et al., 2001) similar to that of ColIII-FGFR31^{ach} and ColIII-FGFR3^{ach} transgenic mice. In this mouse mutant, altered FGFR2 splicing is thought to activate FGFR2 signaling inappropriately in developing mesenchymal condensations.

FGF receptors regulate chondrification and define the boundary of skeletal elements

FGFs have the ability to induce the transition from mesenchyme to chondrocytes in vitro (Richman and Crosby, 1990). However, the role of FGFR signaling in chondrification in vivo is poorly understood. *Fgfr1* is expressed in loose mesenchyme and in condensed pre-cartilage mesenchyme. By contrast, *Fgfr2* is expressed only in mesenchymal condensations. After condensed mesenchyme undergoes chondrification, *Fgfr3* is expressed in proliferating growth plate chondrocytes, *Fgfr1* is expressed in hypertrophic chondrocytes, and both *Fgfr1* and *Fgfr2* are expressed in the perichondrium and periosteum (Delezoide et al., 1998; Orr-Urtreger et al., 1991; Peters et al., 1992; Szebenyi et al., 1995).

We show that joint loss in ColIII-FGFR3^{ach} and ColIII-FGFR31^{ach} transgenic mice results from expanded chondrification, providing in vivo evidence that FGFR signaling promotes chondrocyte differentiation from condensed mesenchyme. The initial step in joint morphogenesis is the specification of the presumptive joint position in a continuous condensed mesenchymal rod. The presumptive joint position is marked by the appearance of a zone expressing low levels of type II collagen juxtaposed by cartilaginous elements expressing higher levels of type II collagen (Fig. 9). As joint development progresses, type II collagen expression is abolished in the joint space. This suggests that cells in the presumptive joint space may elaborate anti-chondrogenic signal(s) that antagonize the chondrification process. This hypothesis is supported by the identification of *Wnt14* expression specifically in the joint interzone (Hartmann and Tabin, 2001). In chick, *Wnt14* is sufficient to suppress chondrification in micromass mesenchymal cell culture. In ColIII-FGFR3^{ach} and ColIII-FGFR31^{ach} transgenic mice and in

Apert syndrome, activation of FGFR signaling promotes chondrification. As a result of overactivation of FGFR signaling, the balance between the anti-chondrogenic signal (WNT14) and pro-chondrogenic signal (FGFR) becomes disrupted. It is likely that this antagonistic signaling in the chondrification process is a unique property of WNT14, as other WNT family members promote chondrification (Hartmann and Tabin, 2001). In addition, the BMP family member, GDF5, expressed in the presumptive joint space, could also restrict the FGF signaling domain. Antagonistic relationships between FGFs and BMP family members are well established in several systems (Neubuser et al., 1997; Weaver et al., 2000). The development of chondrocytes and the perichondrium are closely coupled. Previous studies have revealed that overactivating FGFR3 signaling in chondrocytes resulted in decreased proliferation and reduced *Bmp4* expression in the perichondrium (Naski et al., 1998). Thus, it is not surprising to see loss of *Gdf5* expression in the perichondrium as well. This model also predicts that chondrocytes surrounding the presumptive joint space elaborate a signal that negatively regulates either *Wnt14* or *Gdf5* in the joint space and *Gdf5* in the perichondrium.

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