A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos

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We have generated the first mouse model of fibroblast growth factor receptor 3 (Fgfr3) with the K644E mutation, which accurately reflects the embryonic onset of a neonatal lethal dwarfism, thanatophoric dysplasia type II (TDII). Long-bone abnormalities were identified as early as embryonic day 14, during initiation of endochondral ossification. Increased expression of Patched (Ptc) was observed, independent of unaltered expression of parathyroid hormone-related peptide (PTHrP) receptor and Indian Hedgehog (Ihh), suggesting a new regulatory role for Fgfr3 in embryos. We demonstrate that the mutation enhances chondrocyte proliferation during the early embryonic skeletal development, in contrast to previous reports that showed decreased proliferation in postnatal-onset dwarf mice with activating Fgfr3 mutations. This suggests that signaling through Fgfr3 both promotes and inhibits chondrocyte proliferation, depending on the time during development. In contrast, suppressed chondrocyte differentiation was observed throughout the embryonic stages, defining decreased differentiation as the primary cause of retarded longitudinal bone growth in TDII. This model was successfully crossed with a cartilage-specific Cre transgenic strain, excluding the lung as the primary cause of lethality.

INTRODUCTION

Fibroblast growth factors (FGFs) are a family of polypeptides that play important roles in cell growth, differentiation, survival and in numerous developmental processes (1,2). There are at least three types of membrane-associated FGF binding protein, including the high-affinity membrane-spanning receptor, FGFRs, low-affinity heparan sulfate proteoglycans (HSPG), and a cysteine-rich FGF receptor (CFR). FGFRs are receptor-type tyrosine kinases that bind extracellular FGF ligands, converting these signals into intracellular phosphorylation cascades (3). This receptor family consists of four members encoded by independent genes, each of which produces multiple alternatively spliced variants. The functions of FGFRs in skeletal development have recently attracted considerable attention, as numerous mutations in these genes have been identified in human skeletal dysplasias (4). Mutations in FGFR3 cause short-stature with graded severity, including achondroplasia (ACH), hypochondroplasia (HCH) and thanatophoric dysplasia (TD) (5). In vitro studies have shown that these mutations activate the tyrosine kinase activity of the receptor (6–8). One form of neonatal lethal dwarfism, TDII (OMIM 187600), is caused by a K650E mutation in FGFR3. This mutation is located in the activation loop of the kinase domain and causes constitutive activation (6,7).

Recently, analysis of mouse ACH models with FGFR3 mutations, G380R (9–11), G375C (12) and K650E (13) has shown that both proliferation and differentiation of chondrocytes are involved in retarded bone growth. Protracted differentiation of growth plate chondrocytes was demonstrated in postnatal and adult bones of these mice. Chondrocyte proliferation was inhibited as demonstrated by in vivo incorporation of bromodeoxyuridine (BrdU) or [3H]thymidine (9,12,13), indicating that FGF signals are inhibitory in growth plate chondrocytes. In contrast, studies using transfected cells (6,14), chondrocyte cultures (15) and a rat calvaria-derived, clonal chondrogenic cell line, C5.18 (16), showed enhanced proliferation. Recent studies using a rat chondrosarcoma (RCS) cell line and primary murine chondrocytes indicated that the inhibitory effects were cell-type dependent and required the transcription factor, Stat1 (17).

Long bones in higher vertebrates develop through a process called endochondral ossification, which involves multiple steps of conversion from an initial cartilage template into bone (18,19). The cartilage templates are formed by condensation of mesenchymal cells, which later differentiate into chondrocytes through subsequent, but distinct, steps of proliferation, maturation and hypertrophy. The bone collar, the first bone tissue,
appears by means of intramembranous ossification within the perichondrium surrounding the diaphysis. Within the bone collar, chondrocytes begin to degenerate, forming a primary ossification center. The last step of chondrocyte differentiation takes place in the hypertrophic zone of the growth plate. Here, cells undergo apoptosis and leave calcium deposits, followed by the invasion of osteoprogenitor cells and bone capillaries. The osteoprogenitor cells proliferate and become osteoblasts to synthesize bone over the calcified cartilage matrix, replacing the hypertrophic zone with trabecular bone. Bone resorption by osteoclasts occurs at the ossification center, resulting in a hollow marrow cavity. Thus, longitudinal bone growth occurs through a complex of highly coordinated activities of several cell types. At least two signaling systems are involved in the regulation of bone growth, feedback regulation by parathyroid hormone-related peptide (PTHrP)–Indian hedgehog (Ihh) (20,21) and signaling throughFGFR3.

FGFR3 functions as a negative regulator of bone growth because its targeted disruption results in postnatal-onset skeletal overgrowth (22,23). However, prenatal skeletal development is involved in TDII pathology, suggesting a role for FGFR3 in early embryonic development. The timing of the TDII abnormalities which first appear in skeletal development is unknown, because human samples at less than 12–16 weeks of gestation are not available for analysis. A previously described animal model of FGFR3 with a cDNA ‘knock-in’ K650E mutation resembles ACH, a milder form of dwarfism, due to a reduced level of expression of the mutant allele (13).

In order to investigate the roles of FGFR3 in early stages of skeletal development and to understand the mechanisms of TDII pathophysiology, we have generated mice with an Fgfr3-K644E mutation using an in vivo Cre-loxP recombination strategy to overcome lethal effects caused by this mutation. Mutant pups heterozygous for the mutation showed phenotypes in the embryonic stages with similar timing to human TDII. Here we show that the mutation causes enhanced proliferation of growth plate chondrocytes during the early stages of endochondral ossification [embryonic day 15 (E15)], in contrast to previous observations in adult dwarfism models. In addition, decreased chondrocyte differentiation was observed throughout long-bone development which may account for the retarded longitudinal bone growth of the mutant mice. Tissue-specific expression of the mutant Fgfr3 was tested by crossing with Col2a1-Cre transgenic mice. Our result excluded the lung as a primary cause of lethality at birth in the mutant mice.

RESULTS

Generation of an Fgfr3-K644E mouse with an embryonic phenotype

Reduced Fgfr3 transcription has been reported when a selection marker, the neomycin-resistance gene (neo), is present in the locus (10,12). Taking advantage of this finding, we designed a strategy where neo is used to suppress expression of an Fgfr3 allele with a K644E mutation (corresponding to K650E in humans) (Fig. 1A). The mutation was co-transferred with neo that was flanked by two loxP sites, through homologous recombination in ES cells, and germline transmission was established. Heterozygous mice carrying the targeted mutation with neo (Fgfr3<sup>K644E<sub>neo</sub></sup>) were phenotypically normal and fertile, and served as ‘carriers’. The neo gene was then excised using Cre-loxP recombination by crossing with Ella-Cre transgenic mice (24). This strategy generated mice heterozygous for the mutation (Fgfr3<sup>K644E</sup>), with an expected Mendelian segregation (1:1) at birth (n = 103:94, wild-type and mutant, respectively). Hereafter, we refer to the Fgfr3<sup>K644E</sup> mice as ‘mutants’.

Mutant pups invariably died within a few hours to 1 day after birth (n = 94). Little or no milk was observed in these pups, indicating decreased viability (Fig. 2A). The direct cause of death was not clear from their appearance or behavior. The mutant skeleton of postnatal day 1 (P1) animals showed decreased ossification in the spine, ribs and epiphyses of the long bones, compared with wild-type littermates (Fig. 2B–C). The limbs were shortened in the mutant pups (Fig. 2C and D) with the tibiae and humeri most affected. Lengths of the mutant tibiae/humeri, femurs and ulnae were 77, 88 and 80% of the wild-type, respectively (n = 4–6; P < 0.05). Body weights were not statistically different. In addition, the mutant mice showed features including curvature of the axial skeleton, marked bowing of the ulna and tibia, and a notable round head, which may reflect the macrocephaly seen in human patients. The mutant rib cage was smaller than wild-type and showed marked caudal widening. Widening of limb long bones (Fig. 2C and D), costal cartilage and sterni were also observed in the mutant (Fig. 7A and B). Introduction of a loxP site alone as control strain did not lead to this phenotype (data not shown), indicating that the phenotype was specifically caused by the Fgfr3-K644E mutation. Taken together, the mutant mice show...
Figure 2. Mice heterozygous for the mutation (*Fgfr3*+/K644E) showing characteristics mimicking human TDII. (A) Morphology of the pups (P1). The mutant mice (right) died within a few hours of birth. The precise cause of death was not identified. (B) A mutant skeleton (below) showing decreased ossification in the spine, ribs and epiphyses, compared with a wild-type littermate. Curvature of the axial skeleton and a smaller and caudally widened rib cage are obvious in the mutant. A notable round head in the mutant may reflect the macrocephaly seen in human patients (arrows). Hands and digits were less obviously affected. Forelimb (C) and hindlimb (D), showing marked bowing of the mutant (below) ulna, humerus and tibia, are shown. Shortening of the ossified zone was clearly seen (bars). (B–D) Alcian Blue (cartilage)/Alizarin Red (bone) staining.

Figure 3. Abnormal long-bone growth plates in the mutant mice at P1. (A and B) Sections of the distal portion of femurs. The mutant long bones were shorter than wild-type. Note that the ossified zones were particularly shortened in the mutant long bones, whereas the length of growth plates are similar (thin and thick bars indicating the length of bones from the mid-point and the ossified part, respectively). (C and D) Magnified view of the growth plates. The mutant growth plates were disorganized: resting chondrocyte-like cells were randomly located in the proliferation and hypertrophic zones. Some of them were directly facing the zone of ossification (arrowheads in (D)). Thick and extended perichondrium was observed in the mutant long bones [asterisk in (D)]. The area of expression of chondrocyte markers, PTHrP receptor (G and H), as well as *Ihh* (I and J), type II collagen (K and L) and type X collagen (data not shown), were according to the appearance of chondrocytes (E and F) and their levels were similar in wild-type and the mutant growth plate. Activity of an osteoclast marker, Trap (stained red), was up-regulated in primary spongiosa region of the mutant growth plates (K and L). (A–F) H&E stain; (G–J) in situ hybridization. (A, C, E, G, I and K) wild-type (wt); (B, D, F, H, J and L) mutant (+/K644E) distal femur at P1 (E18.5). Scale bar: 500 μm (A and B); 100 μm (C, D, K and L); 200 μm (E–J).

Characteristics similar to TDII, including dominant inheritance, neonatal lethality and morphological changes in the skeleton.

**Disorganization of the mutant long bone growth plates at P1**

We first examined the histology of the P1 mouse long bones. Shortening of the long bones was also obvious from the histology (Fig. 3A and B). The ossified zone of diaphysis was particularly shortened in the mutant, whereas the height of the whole epiphysis including epiphyseal growth plate was similar. Abnormal differentiation of the mutant growth plates was observed, either as very short or no 'stacked-cell' columnar organization of proliferating chondrocytes (Fig. 3C and D). Resting chondrocyte-like cells with small and round appearance were randomly located in the proliferation and hypertrophic zones, some of which were directly facing the ossification front. To confirm the identity of these cells, we examined the expression of chondrocyte markers and key regulators of bone growth by RNA *in situ* hybridization. The expression of genes, including *Fgfr3*, collagen type II and collagen type X, was detected in accordance with the appearance of chondrocyte types, and no changes in the intensity of hybridization were observed (data not shown). Expression of *PTHrP receptor* (Fig. 3G and H) and *Ihh* (Fig. 3I and J) was detected in the expected area of maturing chondrocytes. In addition, no difference in the expression level was observed between wild-type and mutant growth plates, indicating that the transcriptional regulation of these molecules is not involved in the abnormal phenotype at P1. TUNEL assays showed no significant difference in apoptosis in the growth plate chondrocytes of wild-type and mutant E18.5 mice (data not shown).

**Differentiation of growth plate chondrocytes is suppressed in the mutant starting at early embryonic stages**

Bone growth is a complex process, involving many factors and cell lineages that closely influence each other, including chondrocytes, osteoblasts and osteoclasts. The disorganization of the growth plate observed at P1 may be caused by multiple factors. Identifying the time point when the initial abnormalities in the mutant growth plates appear may help to clarify some of the factors that influence the phenotype. Therefore, we
examined the skeletal development of Fgfr3+/K644E mice in embryos. In mice, endochondral ossification begins at E14–15 with primary ossification centers forming after mesenchymal condensation at E12 (25). The long-bone lengths of the mutant and wild-type mice were not significantly different at E14–15, although the diameters of the mutant bones were significantly increased (Fig. 4A and C, and see below). Initial bone collar formation was also not affected by the mutation, as clearly demonstrated by periosteal expression of the osteoblast markers, osteopontin (Fig. 4E and H) and osteocalcin (data not shown). However, striking differences were observed in the medial regions of the long bones. The hypertrophic chondrocytes showed incomplete maturation without full enlargement (Fig. 4B and D) and a significantly reduced area of osteopontin expression (Fig. 4H) compared with wild-type. In the mutant growth plates, there was no obvious formation of columnar cell stacks of proliferating chondrocytes (Figs 4I and 5B). Chondrocytes in the resting and proliferating zones were smaller and had a ‘tightly packed’ appearance. This was also demonstrated by increased numbers of cells in the mutant growth plates, which had 15–20% more cells than the wild-type (Table 1). These results suggest that long-bone abnormalities in the mutant mice begin as early as E14 during the initiation of endochondral ossification. In addition, chondrocyte differentiation from the resting to proliferating, and to hypertrophic states, was suppressed by the constitutive activation of Fgfr3.

While the area of osteopontin expression was reduced, an increased expression was observed in the mutant growth plates at E15.5 (Fig. 4G and J) and at E18.5 (data not shown), indicating increased osteoblast activity. Tartrate-resistant acid phosphatase (Trap) staining, an osteoclast maker, was also up-regulated in the mutant ossification centers at E15.5 (data not shown) and more pronounced in the primary spongiosa at P1 (Fig. 3K and L). However, increased osteoblast and osteoclast activities may be a secondary effect associated with reduced ossification caused by the suppression of chondrocyte differentiation in the mutant growth plates. The medial portion of the mutant growth plates curved toward the diaphysis into primary spongiosa, whereas in wild-type mice it formed a straight transverse line at E15.5 (Fig. 4E and H). This abnormality is also observed in the mutant long bones at P1 (Fig. 3B). The appearance of thick and extended perichondrium in the P1 mutant growth plates (Fig. 3D) may be due to the position of the ossification front that is pushed toward the diaphysis in the mutant long bones, caused by the protracted differentiation of the growth plate chondrocytes.

**Patched (Ptc) expression is up-regulated in the mutant growth plate and perichondrium at E15.5**

Similar levels of Fgfr3 mRNA in wild-type and the mutant mice were confirmed by a northern blot (Fig. 1C) and in situ hybridization of the growth plates (Fig. 5G and H). At E15.5, Fgfr3 expression was observed widely from the resting to maturing zones with increasing intensity toward the hypertrophic zone. Fgfr3 expression was not limited to the proliferating chondrocytes (23) or the resting zone (26). Next, we attempted to identify molecules responsible for the observed suppression of chondrocyte differentiation in the mutant growth plates. The expression of molecules known to regulate long bone growth was examined by in situ hybridization. Similar to the results at P1, few differences were observed at E15.5, including expression of a chondrocyte marker, Collagen type X (data not shown), and the key regulators of bone growth, PTHrP receptor and Ihh (Fig. 5C–F). On the other hand, the expression of Ptc, a downstream signaling molecule of Ihh and PTHrP receptor, was up-regulated in the mutant growth plates, perichondrium and at the primary spongiosa. Its expression domain was also diffuse and extended into the epiphyses (Fig. 5I and J).

Endogenous Ptc expression was initially found in the perichondral region flanking the Ihh expression domain but not in the cartilage core where Ihh is expressed (20). Based on this, it was suggested that the natural target of the Ihh signal is the perichondrium and not the cartilage core (20). Further study showed that the Ptc expression was also found in proliferating chondrocytes. The strongest Ptc expression was in cells adjacent to Ihh-producing maturing and hypertrophic chondro-

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**Table 1. Enhanced proliferation of the growth plate chondrocytes in E15.5 mutant embryos**

<table>
<thead>
<tr>
<th>Embryonic day</th>
<th>Genotype</th>
<th>Silver grain numbers*</th>
<th>Grain numbers</th>
<th>P-value</th>
<th>Number of cells</th>
<th>Proliferation index*</th>
<th>Proliferation index</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>E15.5</td>
<td>wild-type</td>
<td>15.0 ± 5.9</td>
<td>55.0 ± 9.7</td>
<td></td>
<td>proliferating 4.2 ± 1.3</td>
<td>total 57.2 ± 7.8</td>
<td>0.0746 ± 0.019</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/K644E</td>
<td>19.9 ± 4.0</td>
<td>65.8 ± 5.1</td>
<td>&lt;0.05</td>
<td>proliferating 9.2 ± 2.0</td>
<td>total 65.8 ± 5.1</td>
<td>0.140 ± 0.029</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>E18.5</td>
<td>wild-type</td>
<td>25.9 ± 5.5</td>
<td>45.2 ± 9.7</td>
<td></td>
<td>proliferating 5.6 ± 1.9</td>
<td>total 45.2 ± 9.7</td>
<td>0.124 ± 0.038</td>
<td></td>
</tr>
<tr>
<td></td>
<td>+/K644E</td>
<td>24.9 ± 4.5</td>
<td>55.0 ± 5.7</td>
<td>&lt;0.05</td>
<td>proliferating 6.7 ± 2.3</td>
<td>total 55.0 ± 5.7</td>
<td>0.121 ± 0.039</td>
<td>n.s.</td>
</tr>
</tbody>
</table>

n.s., not significant.

*Numbers of silver grains, indicating newly synthesized DNAs, were counted in proliferating cells (n = 16). Background grain numbers, counted in the equivalent area, were then subtracted.

*bProliferation index was calculated as the ratio of proliferating cells that had incorporated radiolabeled thymidine, to the total cell number present in each of three given areas of the three sections of distal femurs (n = 9 in total), randomly chosen in resting and proliferating zones.
cytes and decreased toward the end of the bones at E17.5 (27). Our data confirm the latter pattern of Ptc expression at E15.5 in mouse growth plates. In wild-type perichondrium, Ptc expression corresponds to the areas flanking Ihh (Fig. 5). At the zone of maturing chondrocytes, Ihh and Ptc are expressed adjacent to each other and barely overlapping. In addition, the epiphyseal end of Ptc expression corresponds to the beginning of the proliferating zone. The relationship between Ihh and Ptc expression patterns was somewhat distorted in the mutant growth plates. Ptc was expressed in the perichondrium corresponding to the area where Ihh was expressed, but it was more intense. In the growth plate core, Ptc expression was observed toward the hypertrophic chondrocytes and almost overlapping Ihh expression. In the other direction, Ptc was expressed broadly and with decreasing intensity toward the epiphyseal end. This coincides with the lack of apparent proliferating columns in the mutant growth plates. Persistent expression of Ptc in the epiphyseal end of growth plates resulted in broader regions of overlap with Fgfr3 expression. At a later stage (E18.5), Ptc expression was similar in wild-type and mutant growth plates.

Proliferation of the mutant growth plate chondrocytes is increased at E15.5

The most remarkable difference in E14.5 long bone of the mutant mice versus controls was increased bone width and thicker layers of undifferentiated chondrocytes in the mutant growth plates (Fig. 4A and C). There are at least three possible explanations for these phenomena. First, apoptosis of the chondrocytes may be inhibited in the mutant; secondly, slower differentiation may lead to accumulation of undifferentiated cells over time; thirdly, chondrocyte proliferation may be up-
regulated. No significant difference was observed in apoptosis of the growth plate chondrocytes of E15.5 wild-type and the mutant mice, using TUNEL assay (data not shown). Because differentiation in the primary ossification center starts around E14.5, it is unlikely that decreased chondrocyte differentiation led to significant accumulation of undifferentiated chondrocytes in the mutant growth plates. Thus, we speculated that chondrocyte proliferation is up-regulated. To measure directly the effects of the mutation on chondrocyte proliferation, we examined the incorporation of [3H]thymidine into DNA during the S phase in vivo at E15.5 and E18.5. At E15.5, the labeled cells were found scattered in the resting and maturating zones (Fig. 4K and L). This is in contrast to the labeling patterns observed in the proliferating zones of adult stages (such as 2 weeks and older) (9,12,13,23). A proliferation index was calculated as a ratio of proliferating cells that had incorporated radiolabeled-thymidine to the total number of cells, present in given areas of the growth plate. Indeed, chondrocyte proliferation in the mutant growth plates was twice (188%) that of wild-type at E15.5 (Table 1). However, at E18.5, little difference was observed (97.6%).

Ligand-independent proliferation was demonstrated in a BaF3 cell line stably expressing FGFR3 with the K650E mutation (6). Nonetheless, the mitogenic response of cells representing the conditions of a dominant disorder, where wild-type and the mutant Fgfr3 are co-expressed, have never been assessed. To investigate this issue, we derived mouse embryonic fibroblast (MEF) cells from E14.5 litters. Consistent with the previous report, the mutant cells showed increased proliferation compared with wild-type in the absence and presence of FGF1 and the Fgfr3-specific ligand, FGF9 (Fig. 6A). Higher ligand concentrations increased the proliferation of both wild-type and mutant cells. Activation of Map kinase in cells expressing the mutant Fgfr3 has been reported (15,17). Basal

Figure 6. FGF signaling in MEF cells derived from wild-type (wt) and the mutant mice (+/K644E). (A) Proliferation assay. Increased proliferation in the mutant MEF compared with wild-type was observed in the absence of the ligand. Proliferation of the mutant cells was increased further by the addition of ligand as in the case of wild-type cells. (B) FGF treatment stimulated phosphorylation of Map kinase in both wild-type and the mutant MEF. Sustained phosphorylation was observed in the mutant cells on treatment with FGF1 and, particularly, with an FGFR3-specific ligand, FGF9. na, no addition of ligand. Cells were ligand-induced for 2, 5, 30, 60 and 120 min. Phosphorylation was normalized against the amount of Map kinase protein and shown in the graph below.

Figure 7. The rib cage and the lung phenotypes in the mutant pups at P1. (A and B) Thoracic cages. Alcian Blue/Alizarin Red staining. The mutant mice showed no ossification in the sternum; instead, thickening of the cartilage and bifurcation was observed. (C and D) Sections of the sternabrae between the fifth and sixth costal cartilage; (E and F) the fifth costal cartilage. (G and H) [3H]thymidine incorporation in E18.5 costal cartilage (fifth). (I and J) Lungs at E18.5; (K and L) lungs at P1. The morphology of the lungs was similar in wild-type and mutant at E18.5. Reduced alveoli were formed in the mutant lung at P1. (C–H) H&E stained sections. (A, C, E, G, I and K) wild-type (wt); (B, D, F, H, J and L) mutant (+/K644E). Bar: 200 µm (G and H); 100 µm (C–F and I–L).
levels of Map kinase activity are similar in wild-type and the mutant cells (Fig. 6B). On FGF treatment, Map kinase activation was observed in both wild-type and the mutant cells. In addition, sustained Map kinase activation was observed in the mutant MEFs at 2 h of ligand treatment (Fig. 6B).

The lung is not the primary cause of lethality in the mutant mice

TDII patients are known to die at birth because of respiratory failure (28). However, it is unclear whether the respiratory failure is primarily caused by lung abnormalities, or by collapse of the thoracic cavity owing to the abnormal formation of the rib cage, or by other unknown causes. Fgfr3 is highly expressed in the lung (29) and its expression increases towards birth with a peak in the days after birth (P2–P10 in rat) (30). Both Fgfr3 and Fgfr4 have been reported to play an essential role in adult lung alveogenesis. The lungs of Fgfr3/ Fgfr4 double knockout mice fail to form secondary septae during the postnatal period (29). The functions of Fgfr3 in embryonic lungs are unknown. To determine whether the lung is affected by the overactivation of Fgfr3, we first examined the morphology of the perinatal lungs. The lungs of wild-type pups after birth showed the formation of large alveoli (Fig. 7K). However, in the mutant mice alveoli were not completely formed (Fig. 7L). Lungs of E18.5 or earlier stages showed no morphological differences between the mutant and wild-type pups (Fig. 7I and J). There were no significant differences in the proliferation of lung tissue measured by incorporation of [3H]thymidine at E18.5 and P1 (data not shown). Subsequent to alveogenesis, Fgfr3/Fgfr4 double mutant lungs failed to shut off synthesis of elastin, a gene known to be important for lung architecture, which indicated that Fgfr3 and Fgfr4 function to down-regulate elastin deposition (29). The ligand FGF2 has also been shown to decrease elastin gene transcription (31). However, there was no significant difference in elastin expression in Fgfr3+/K644E mice by Elastin–Van Giemsa staining (data not shown). These observations suggested that the lung abnormalities in Fgfr3+/K644E mice are different from that of Fgfr3/ Fgfr4 double mutant mice.

The failure in alveoli formation in P1 lungs of Fgfr3+/K644E mice could be caused by ineffective respiratory movements, which could be a secondary consequence of abnormal rib cage formation. Therefore, we examined the rib cages of our mice. Skeletal staining of wild-type pups showed ossifications in all sternum and upper regions of the xiphoid process at P1 (Fig. 7A). In contrast, no ossification of sternum was observed in the mutant pups. This feature was accompanied by widening of the sternum and costal cartilage (Fig. 7B). The histology of the mutant costal cartilage revealed that chondrocytes were ‘tightly packed’ in appearance, similar to the resting zone of the mutant growth plates at E15.5 (Fig. 7F). Proliferation was assessed in the P1 costal cartilage using in vivo incorporation of [3H]thymidine, and a proliferation index was calculated. Indeed, proliferation in the mutant costal cartilage was increased by 258% compared with wild-type mice at E18.5 (proliferation index of the mutant, 0.132 ± 0.0487, as compared with that of wild-type, 0.0512 ± 0.0106, P < 0.05). Increased chondrocyte proliferation observed in the costal cartilage of the mutant pups is consistent with our data growth plate data. In addition, little difference was observed in chondrocyte proliferation of the costal cartilage at an earlier stage (E15.5), suggesting that the timing of chondrocyte proliferation may be tissue specific.

We tested the possibility that abnormal rib cage formation may prevent the mutant pups from efficient breathing, and attempted to determine whether the lung abnormality is directly caused by the overactivation of Fgfr3. One advantage of Cre-loxP technology is that tissue-specific Cre transgenic mice allows expression of the mutation in selected tissues. Therefore, we crossed our carrier mouse (Fgfr3+/K644E) with Col2a1-Cre transgenic mice (32). These mice allow Cre recombinase to be expressed under the control of the promoter of collagen type II, the principal protein in cartilage. Absence of the mutant Fgfr3 expression in the lung would demonstrate any abnormality in the mutant lung when crossed with Col2a1-Cre mice should be secondary to the effect of the mutation in cartilage. The mutant pups obtained using Col2a1-Cre mice showed skeletal phenotypes similar to the offspring obtained by the cross with Elia-Cre mice (data not shown). The majority of the mutant offspring produced from the Col2a1-Cre cross died on day 1, similar to those produced from the Elia-Cre cross. The P1 lungs of the mutant pups showed reduced alveoli formation (data not shown), indistinguishable from the phenotype observed in the Elia-Cre cross. This result strongly suggests that the morphological abnormality observed in the mutant lungs is not primarily caused by the overactivation of Fgfr3 and excludes the lung as a primary cause of neonatal death. The lethality at birth is most likely caused by malformations of the sternebrae and the costal cartilage.

DISCUSSION

Quantitative differences in ligand-independent Fgfr3 activation is one factor affecting the severity and age of onset of skeletal phenotypes. In our study, embryonic onset was achieved by designing a strategy using Cre-loxP technology allowing expression of highly activated Fgfr3 with the K644E mutation. This strategy enabled us to investigate the abnormal effects of the Fgfr3-K644E mutation in the mouse embryo. The embryonic abnormalities may be caused by the over-activated tyrosine kinase activity of the Fgfr3-K644E or by abnormal downstream signaling pathways triggered as a result of the mutation. This model also gives the opportunity to investigate the possible roles of Fgfr3 in embryonic stages that have not been assessed in previous studies with Fgfr3 null mice that are normal at birth, possibly owing to redundancy of other members of the gene family (22,23,29).

Bone formation is a complex process that involves proliferation and differentiation of chondrocytes, as well as osteoblast and osteoclast activities that enlarge and shape the bone matrix. Bone development involves the stages of patterning, mesenchymal condensation, bone formation and growth, followed by bone remodeling. In this study, we investigated the early stages of bone formation taking place in the embryonic period, in an attempt to identify the primary cause of long-bone shortening in TDII. We have now demonstrated that several factors affecting bone growth are not the primary cause of the shortened limbs. Neither is timing of endochondral ossification or initial bone formation (i.e. bone collar formation) affected by this mutation. Bone forming activity assessed by osteopontin and osteocalcin expression, and bone resorption...
activity by trap, appeared to be up-regulated in the mutant mice. The effects of FGFs in osteoblast and osteoclast activities have been reported previously (33,34). However, whether Fgfr3 plays a role directly in activating these cells is not clear and is open for further investigation. We have shown that the K644E mutation causes enhanced proliferation of growth plate chondrocytes during a limited time in early stages of endochondral ossification (E14–15). However, at a later gestational age (E18), the mutant chondrocytes proliferate at rates similar to wild-type. In contrast, decreased chondrocyte differentiation continued to be observed throughout long bone development. This uncoupling of proliferation and differentiation favors the interpretation that only suppression of differentiation could account for the retarded longitudinal bone growth in the mutant mice. Taken together, the data suggest that the K644E mutation causes significant reductions in embryonic ossification leading to retarded longitudinal bone growth by suppressing chondrocyte differentiation at the earliest stages of endochondral ossification. The widened bone in the initial stage may indirectly contribute to decreased medial differentiation by diminishing adequate amounts of periostal signaling factors. It is possible that the cause of shortened limbs in the Fgfr3-K644E mutant mouse may be different from other mouse models of activated Fgfr3 where the phenotype appears later in the postnatal stage.

The various forms of short-limb dwarfism may be caused by alterations in diverse genes. However, their affected sites and appearance of the dwarf phenotype are distinct. Overexpression of PTHrP leads to a completely cartilaginous endochondral skeleton due to a delay in chondrocyte differentiation (35). Expression of constitutively activated mutant PTHrP receptor showed delayed mineralization, decelerated conversion of proliferative chondrocytes into hypertrophic cells and prolonged presence of hypertrophic chondrocytes (36). In contrast, a null mutation of PTHrP and its receptor exhibited premature maturation of chondrocytes leading to excessive bone formation with short limbs (37,38). Ihh is expressed in maturing chondrocytes and induces expression of PTHrP. Increased PTHrP signaling suppresses chondrocyte maturation, thereby establishing a negative feedback loop (20). Animals with a targeted null mutation of Ihh display markedly reduced chondrocyte proliferation, abnormal maturation, and absence of osteoblast development (39). However, the levels of expression of these molecules were unaltered in our model, suggesting that they do not participate in the TDII pathology. In this study, up-regulation of Ptc was observed in E15.5 mutant growth plates. Ptc is a receptor for Ihh, and its own signaling controls its expression (40,41). Heterozygous Ptc null mice are reported to be larger; however, only some of them had hindlimb defects, such as extra digits and syndactyly (42). Whether increased expression of Ptc directly causes the limb phenotype in our mutant mice and how overactivated Fgfr3 involves Ptc in parallel to Ihh expression are unknown. These questions need to be addressed and are currently under investigation.

Postnatal-onset dwarf mice with activating Fgfr3 mutations showed inhibition of chondrocyte proliferation (9,12,13). In contrast, this study demonstrates that overactivated Fgfr3 enhances proliferation of growth plate chondrocytes in the early embryonic skeletal development. These results suggest that signaling through Fgfr3 can both promote and inhibit chondrocyte proliferation depending on the time during animal development. Cell-type dependent dual effects of FGF on proliferation have been reported in various cells, including breast cancer cell lines (43,44). This study highlights the observation that FGF signaling, via Fgfr3, regulates chondrocyte proliferation in opposite ways at different stages of development. One possible mechanism for this biphasic effect may be a switch in signaling molecules downstream of the abnormal receptor activation. While STAT proteins may be important for postnatal inhibitory signaling, other signaling factors such as Map kinase may be required for the embryonic proliferative response. Local ligand expression may also regulate the response of mutant cells. Although autophosphorylation of Fgfr3 in the presence of the K644E mutation occurs constitutively (6–8), the addition of ligand further increases the mitogenic response (6). FGF1, FGF2 and FGF9 bind Fgfr3 with relatively high affinity (45); however, the ligands of Fgfr3 in vivo and their downstream effects in individual tissues have not been precisely defined. The possibility of the limited availability of ligands in postnatal bone has been proposed previously (9).

Abnormal Stat1 activation and p21\(^{CIP/WAF}\) induction by the K650E mutation has been suggested to be responsible for suppression of chondrocyte proliferation in TDII (46). Activation of Stat1 in cell cultures (15,17) and Stat5a/Stat5b in the adult Fgfr3 mutant animal (13) have also been reported. However, activation of Stat proteins was not detected in the growth plates of E15, E18 and P1 adults generated in this study (data not shown). Furthermore, introduction of a p21 null background (47) did not rescue the TDII phenotype (data not shown). Because Stat1, Stat5a or Stat5b knockout mice showed no significant prenatal skeletal phenotype (48–51), we speculate that the timing of the TDII appearance of phenotype may account for our findings on STATs activation. FGF treatment of bone organ cultures inhibited growth of wild-type bone, but no inhibition was observed in bone culture from Stat1\(^{−/−}\) mice. This result indicates that the inhibition of bone growth by FGF requires Stat1 (17). In the same study, a Stat1\(^{−/−}\) bone rudiment cultured in the presence of FGF showed increases in length and width, which is consistent with our observation of increased chondrocyte proliferation and the absence of Stat1 activation. Abnormal STAT activation may play a larger role in postnatal skeletons in mice and in human fetal bones, which are in the stages comparable to that of postnatal development in mice. Similarly, the timing of the appearance of the TDII phenotype may also account for some of the differences in the previous observations on the down-regulation of the Ihh pathway (52).

Recently, vertebrate Sprouty and Perlecan have been suggested to play roles in Fgfr3-related skeletal disorders (53,54). Other molecules downstream of aberrant signals from the mutant Fgfr3 may be identified by techniques including a cDNA expression microarray. This TDII mouse model will be useful for identifying not only the molecular cause of the abnormal signaling associated with the Fgfr3-K644E mutation, but also the possible roles of Fgfr3 in the embryo. The usage of transgenic mice with tissue-specific promotors-driven Cre recombinase will allow expression of the mutant Fgfr3 in selected tissues, opening a door for studying activated FGF signals in tissues/organs of interest. An example of this strategy was demonstrated in this study. By using the cartilage-
specific expression of overactive Fgfr3, the lung was excluded as a primary cause of perinatal lethality in our mice. A somatic K650E mutation in FGFR3 was found in bladder and cervix carcinomas (55) and in multiple myeloma (56). The tissue specific expression of the mutant Fgfr3 in such tissues will be useful for the investigation of the role of Fgfr3 in cancer and in other organs.

MATERIALS AND METHODS

Site-directed mutagenesis and targeting vector construction

Recombinant phage clones containing the Fgfr3 locus were isolated from a 129 mouse genomic DNA library (Stratagene, La Jolla, CA). The mutation (underlined) was introduced using PCR primers F2122E (5′-GGCTCGAGATGTGCACAACCT-TTCAGATCTCCCTACCCCCAT-3′) and R2080 (5′-GCCTGAGC-3′). The PCR product was digested with XhoI and AccI and the 181 bp fragment was subcloned into the Smal–SphI 2.3 kb fragment, then into the Smal–HindIII right arm (3.3 kb). The EcoRI–SmaI 3.2 kb left arm was subcloned into the plloxneo vector (57) followed by insertion of the right arm to achieve the final targeting construct (Fig. 1A).

Homologous recombination in ES cells and generation of germine chimeras

TC1 ES cells (23) were transfected with NotI-digested targeting vector DNA and selected with G418 and FIAU as described (58). ES cell colonies resistant to double selection were isolated and subjected to Southern blot analysis. Genomic DNA isolated from the clones and parental TC1 cell line were digested with SpeI or with NotI and EcoRV, size-fractionated, transferred to Nylon membranes and hybridized with the 5′ probe (BamHI–SacI, 1.9 kb) generating 9 kb SpeI (Fig. 1B) and 11 kb NotI–EcoRV fragments (data not shown) in the recombinants. ES cells heterozygous for the targeted mutation were microinjected into C57BL/6 blastocysts, implanted into the uterus of pseudopregnant Swiss Webster (Taconic, Germantown, NY) foster mothers and developed to term. Male chimeras were mated with NIH Black Swiss females (Taconic). Germline transmission was confirmed by agouti coat color in F1 animals, and the offspring were genotyped for the Fgfr3 mutant allele by Southern blot analysis.

Genotype analysis

In vivo excision of neo was achieved by mating Fgfr3+/K650Eneo with Ella-cre mice (a gift from Dr H. Westphal, NIH, Bethesda, MD). Genotypes were confirmed by Southern blot and/or PCR using primers F1676 (5′-GGGTTAGCTGTTTGCCGAC3′) and R2080, followed by BbsI digestion. To detect the neo gene, PCR was performed using primers TW13 (in the neo gene, 5′-CAGCTCTATTCCCTCCACTCATGATT-3′) and R3-36s (in Fgfr3, 5′-CATACAACGTGGGTGCTG-3′). Amplification was with 30 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 1 min.

Northern blot

Total RNA was isolated from the brains of animals at P1 using RNA STAT-60 (Tel-Test, Friendswood, TX). Poly(A) RNA was isolated through one round of column purification with an mRNA purification kit (Amersham-Pharmacia, Piscataway, NJ). Five micrograms of the poly(A) RNA were subjected to northern blot analysis. The Fgfr3 probe was from the 3′-region of the mouse cDNA (nucleotides 2456–3024 of GenBank accession no. M81342).

Skeleton staining, histology, in situ hybridization and thymidine incorporation

Whole-skeleton staining with Alizarin Red S and Alcian Blue was performed as described (59). Histologic sections were prepared from selected tissues that were fixed in 4% paraformaldehyde in PBS and paraffin embedded. Sections (8 μm) were either stained with hematoxylin and eosin (H&E) or subjected to in situ hybridization. In situ hybridization was performed using standard procedures. Probes were labeled using an SP6/T7 transcription kit (Boehringer Mannheim–Roche, Indianapolis, IN) with [35S]UTP (1000 Ci/mmol; Amersham-Pharmacia) and unincorporated nucleotides were removed using Quick Spin G-50 Sephadex Columns (Boehringer Mannheim–Roche). Chondrocyte proliferation was assayed in vivo by i.p. injections of pregnant mice with [methyl-3H]thymidine (70–86 Ci/mmol; Amersham-Pharmacia) and killed after 4 h. Tissues were fixed in 10% formalin and processed as above. Immunohistochemistry with anti PCNA antibody (Signet, Dedham, MA) was performed using standard procedures.

MEF proliferation assay and immunoblot

Primary MEF cultures were derived from E14.5 embryos and cultured using standard procedures. Cells (1 × 10⁶/well; passage 2) were plated in 96-well plates in DMEM containing 15% fetal calf serum and allowed to attach. Following depletion in serum-free medium for 12 h, cells were incubated in the serum-free medium containing 10 μg/ml heparin (Sigma, St Louis, MO) and FGF1 or FGF9 (R&D Systems, Minneapolis, MN) at various concentrations (Fig. 6A) for a further 48 h and assayed for proliferation using Cell Titer 96 (Promega, Madison, WI). For immunoblot analysis, cells 80% confluent in 12-well plates were induced with 100 ng/ml ligands for various time points (Fig. 6B), following the depletion as above. One-fifth of MEF whole-cell lysate in the SDS-loading buffer was loaded on 4–15% gradient SDS–polyacrylamide gels. Proteins were transferred onto nitrocellulose membranes, blotted with antibodies specific for phosphorylated Map kinase (E10) and Map kinase (New England BioLabs, Beverly, MA), and detected with ECL (Amersham-Pharmacia). Quantitative analysis was performed using NIH Image (http://rsb.info.nih.gov/nih-image/).

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