Several gain-of-function mutations in a receptor tyrosine kinase, fibroblast growth factor receptor 3 (FGFR3), cause dwarfism in humans. Two particularly severe dwarfsisms, thanatophoric dysplasia type II (TDII) and severe achondroplasia with developmental delay and acanthosis nigricans (SADDAN), are associated with glutamic acid (E) and methionine (M) substitutions at the K650 residue in the kinase domain. TDII is lethal at birth, whereas most of the SADDAN patients survive the perinatal period. However, FGFR3 with the SADDAN mutation is more activated than FGFR3 with the TDII mutation in vitro. To find out whether the K650M mutation also causes the SADDAN phenotype, we introduced the corresponding point mutation (K644M) into the mouse Fgr3 gene. Heterozygous mutant mice show a phenotype similar to human SADDAN, e.g. the majority of the SADDAN mice survive the perinatal period. This suggests that the survival of SADDAN patients is indeed attributed to the K650M mutation in FGFR3. The long bone abnormalities in SADDAN mice are milder than the TDII model. In addition, overgrowth of the cartilaginous tissues is observed in the rib cartilage, trachea and nasal septum. The FGF ligand at the low concentration differentially activates Map kinase in primary chondrocyte cultures from wild-type and SADDAN mice. Comparisons of the molecular bases of the phenotypic differences in SADDAN and TDII mice may increase our understanding of the factors that influence the severity in these two related skeletal dysplasias.

INTRODUCTION

Fibroblast growth factors (FGFs) are a family of at least 23 polypeptides which stimulate arrays of biological responses, including cell growth, differentiation, survival, motility and adhesion (1,2). High-affinity receptors for FGFs, the fibroblast growth factor receptors (FGFRs), are membrane tyrosine kinases. The FGFR family consists of four independent genes that share a highly conserved structure consisting of immunoglobulin (Ig)-like domains, a single transmembrane domain and a tyrosine kinase domain. Upon binding of FGF ligands, the receptor dimerizes and undergoes interchain auto-phosphorylation in the intracellular domain, transmitting signals into the cell (3). FGFR3 is a negative regulator of the bone growth as targeted disruption of the mouse Fgr3 gene causes a skeletal overgrowth (4,5).

FGFR3 has attracted attention since a number of mutations can cause dwarfism in humans. In vitro expression studies have shown that these mutations cause activation of its kinase function (6). Examples of these mutations include: (i) mutations in G380R and G375C in the transmembrane domain which cause achondroplasia (ACH); (ii) mutations in R248C in the linker region between the second and third Ig domain which cause thanatophoric dysplasia (TD) type I; and (iii) mutations in K650E in the tyrosine kinase domain which cause TD type II (7–10). The mechanism of overactivation is speculated to be stabilization of the active receptor dimer by covalent bonding of cysteines (R248C) and by increased charges (G380R) (7,8,11). The graded activation of the tyrosine kinase function could explain the severity of abnormalities found in FGFR3-related dwarfism in humans (7).

A novel mutation in FGFR3, A1949T:K650M, has been reported in four unrelated individuals with a newly recognized non-lethal disorder, severe ACH with developmental delay with acanthosis nigricans (SADDAN) (12,13). Besides the severe skeletal dysplasia, acanthosis nigricans (hyperkeratosis and hyperpigmentation), brain structural anomalies and hearing loss are observed in the surviving SADDAN patients (12). Interestingly, a mutation at the adjacent nucleotide, A1948G: K650E, causes TDII, a lethal form of skeletal dysplasia. In addition, six patients with HCH, a very mild form of dwarfism, have been recently found with G1950T and G1950C (causing the K650N substitution) and A1948C (K650Q) mutations (14). Thus, E, M and N/Q substitutions at the K650 residue correlate with three skeletal disorders with graded severity and distinct morphological and physiological characteristics.

We hypothesized that the difference in severity in the three disorders might be caused by the graded abnormal activation of FGFR3 by the K650 substitution. Unexpectedly, the kinase assay showed that the FGFR3 protein with the SADDAN mutation is more activated than that with the TDII mutation.
in vitro (this study and 13,14), even though the TDII phenotype is more severe than SADDAN in humans. What causes this discrepancy between kinase activity and the physiological phenotypes in these two dysplasias? To clarify this, we have introduced the K644M mutation in mouse Fgfr3 which corresponds to human K650M. We have taken the same Cre-loxp strategy that was used in the TDII mouse model (15), so that direct phenotypic comparison of the two mutant mouse lines is possible. Here we report on the generation and phenotypic analysis of mice with the Fgfr3-K644M mutation. The current study demonstrates that mice carrying one allele of Fgfr3 with the K644M mutation show severe morphological changes in the skeleton but prolonged survival, supporting the observations in human SADDAN. This mouse model provides a basis to understand better the molecular mechanism of how mutations in the Fgfr3 kinase domain lead to severe skeletal dysplasias.

RESULTS

Fgfr3 with the K644M mutation is highly activated in vitro

In order to compare the kinase activities of Fgfr3 with the SADDAN, TDII and HCH mutations, we constructed the expression vectors using the mouse Fgfr3 cDNA. The receptor proteins transiently expressed in 293 cells were a doublet of ~100 kDa (Fig. 1A, blot: Fgfr3) which may represent different glycosylation states of these overexpressed proteins (16). Upon γ-ATP incorporation assay, the highest level of autophosphorylation was observed in Fgfr3 with the K644M (SADDAN) mutation followed by the K644E (TDII) mutation (Fig. 1A). Much lower activation was seen in the K644N (HCH) and wild-type receptors. A higher phosphorylation was observed in the upper band of the doublet. A quantitative analysis, normalized by the amount of Fgfr3 in the reaction, showed that the K644E mutant was 11 times more phosphorylated than that of the K644N and wild-type receptors in the upper band. In addition, the K644M mutant was 1.3- and 1.5-fold more activated than in the K644E mutant in the upper and lower bands, respectively. Next, a blot with an anti-phosphotyrosine antibody, pY20, was performed and blotted with 4G10 (chr: pY20). This blot is to examine the phosphorylation states of the Fgfr3 proteins in cells, while the γ-ATP incorporation assay accesses the abilities of immunoprecipitated Fgfr3 proteins to autophosphorylate. Distinct from the γ-ATP incorporation experiment, the lower band of the doublet was more intensively phosphorylated in the pY20 blot. The lower band of the K644M mutant was 3.3-fold more activated than in the K644E mutant. An overall high phosphorylation of the cellular substrates was also observed in cells expressing the K644M mutant (Fig. 1B) with some particular phosphorylated bands. The 110 kDa phosphorylated band was identified as the Fgfr3 proteins. However, the rest of the phosphorylated proteins remain to be identified.

Taken together, the in vitro kinase assays show that the activation of Fgfr3 proteins are in the order of SADDAN (K644M) > TDII (K644E) >> HCH (K644N). The lower activation of the HCH mutant is consistent with the mild phenotype of this disorder in humans. However, the higher activation of the SADDAN mutant protein compared with the TDII mutant protein does not agree with the milder characteristics of the SADDAN patients. The cause of this discrepancy between the kinase activity and phenotypes of the two dysplasias, SADDAN and TDII, is unknown.

Longer survival of mice with the heterozygous Fgfr3-K644M mutation

Even though the K650M mutation has been associated with SADDAN in humans, only a limited number (four, to date) of individuals with such a mutation have been reported (12,13). Therefore, it was not clear whether the K650M mutation directly caused the milder phenotype of SADDAN, or whether there are other genetic and/or non-genetic influences to affect the otherwise very severe manifestations of the K650M mutation. To address this question, we generated a mouse model with the K644M mutation using the same Cre-loxp strategy that was employed in the TDII mouse model (15) (Fig. 2A). Mice heterozygous for the mutation (Fgfr3+/-K644M) were obtained with an expected Mendelian segregation (1:1) at birth (n = 56; 62, wild-type and mutant, respectively). In contrast to the TDII mouse model, the Fgfr3+/-K644M mutant pups survived the perinatal period. We also observed two distinct groups of offspring with the same Fgfr3+/-K644M genotype within the littermates (Fig. 3A). One group of pups (26 out of 50 mutants (52%)) was very small and died before 4 weeks after birth. Another group was only mildly affected...
and some of them [12 out of 50 (24%)] lived longer than 3 months, including some up to as long as the wild-type controls (Fig. 3A). The difference between the two groups of mutant mice is obvious from the measurement of the body weight and the tail length (Fig. 3B). Some of the mildly affected mutant mice (both male and female) were fertile. However, homozygous mutants were never observed after birth, indicating that mice homozygous for the K644M mutation died during gestation.

To test whether the genetic background may influence the proportion of severely affected mice, we have crossed the fertile Fgfr3+/-K644M mice to wild-type. This produced a higher proportion of the severely affected mice [64 out of 84 mutants (76%)] than the Elia-Cre cross. About 7% (6 out of 84) lived longer than 3 months. We also crossed the fertile Fgfr3+/-K644M males to CD females. About 46% of total mutant mice (13 out of 28 mutants) were severely affected and 14% (4 out of total 28 mutant mice) lived longer than 3 months. This indicates that the genetic background of mice may indeed influence the proportion of severely affected mice. The incomplete neo excision from the carrier mice upon Elia-Cre cross is also possible, even though the neo was not detected after genotyping using PCR on the tail genomic DNA.

The direct cause of death of the mutant mice was not clear from their appearance or behavior. Most of the severely affected mutant mice showed malocclusion (imperfect positioning of the teeth), while the mildly affected group of mice did not. However, we also found that several severely affected mutant mice died without apparent malocclusion. Therefore, the cause of death other than malnutrition could be caused by malocclusion. Taken together we concluded that mice that carry a heterozygous Fgfr3-K644M mutation survive longer than TDII mutant mice, supporting the notion that the K644M mutation indeed causes the SADDAN phenotype. Hereafter we refer to Fgfr3+/-K644M as SADDAN mutant mice.

Mice with the SADDAN mutation have a milder skeletal phenotype than TDII
We have compared the postnatal day (P)1 skeletons of TDII and SADDAN mice (Fig. 3C and D). They show common features; however, the SADDAN mice are more mildly affected than the TDII mice (Fig. 3C). Marked bowing of the ulna and tibia seen in TDII mice were not obvious in the SADDAN mice either at this stage or later (P20) (data not shown). The smaller rib-cage of the SADDAN mutant pups became evident only after P4 (data not shown). The histologic abnormalities of the embryonic and postnatal long bones in the SADDAN mutants are also similar to those in TDII mice, except that in SADDAN mice the differences in the total bone length become recognizable at P1, later than in TDII mice (Fig. 4A and C). The formation of the secondary ossification center was delayed in SADDAN mice (data not shown). Secondary ossification initiates as a blood vessel penetrates from the articular surface into the epiphyses, followed by differentiation of surrounding chondrocytes to hypertrophy. Both wild-type and SADDAN mice show the penetration of blood vessels from the articular surface at P4 (Fig. 4G and H). However, the hypertrophic chondrocytes are sparse and not fully mature in the SADDAN mutant, suggesting that the decrease in chondrocyte differentiation causes the delay in secondary ossification. The ingrowth of mesenchymal tissue across the physis, characteristic morphology often observed in human ACH, was observed in the P17 mutant growth plates (Fig. 4J and L).

Overgrowth of cartilaginous tissues in the SADDAN mutant mice
Fgfr3 is expressed in the hyaline cartilage in the developing and in adult long bone growth plates (4,5,15,17,18). Hyaline cartilage is the most common form of cartilage and contains type II collagen as the major collagen type. In adults, articular cartilage, walls of larger respiratory passages (nose, larynx, trachea, bronchi) and the costal cartilage, are also categorized in this type. A delay in the sternabrae ossification is observed in SADDAN mutant mice at P1, as well as thickening of the costal cartilage (Fig. 5B). This is similar to the phenotype of TDII mice (15), but is much milder. This supports the notion that the malformation of the sternabrae and the costal cartilage

Figure 2. Introduction of the K644M mutation into the mouse Fgfr3 locus. To test whether the mild phenotype of SADDAN in humans is directly caused by the K650M mutation in FGFR3, we generated a mouse model with the corresponding amino acid substitution, K644M. (A) A SADDAN mouse model was generated in two steps. Step 1: the point mutation, K644M (corresponds to human K650M) in exon 15, was co-transferred with a neo gene (flanked by the loxP sequences) inserted in intron 10 through homologous recombination. Mice heterozygous with a targeted mutation with the neo gene (genotype: +/-K644Mneo) were phenotypically normal and fertile, serving as a ‘carrier’. Step 2: the neo gene was removed through an in vivo Cre-lox recombination by crossing with Elia-Cre transgenic mice. The resulting mice (genotype: +/-K644M) showed a phenotype closely resembling human SADDAN. (B) The presence of a 9 kb fragment by Southern blot confirmed the correct targeting (+/-K644Mneo). The removal of the neo gene following crossing with a Cre mouse was confirmed by the absence of the SpeI 9 kb fragment (+/K644M). The presence of the mutation was also confirmed by PCR followed by restriction digestion and sequencing (data not shown). (C) Northern blot analysis of poly(A)+ RNA from P1 brain shows similar levels of transcripts in wild-type and mutant mice. Three examples of the SADDAN mutant mice (+/K644M) are shown.

Human Molecular Genetics, 2001, Vol. 10, No. 12 1257
may cause the lethality at birth in the TDII mice. Histological abnormalities in the costal cartilage growth plates at the junction to the rib-bones are similar to the limb growth plates (Fig. 5G). At 15 months, marked thickening and the clonal proliferation of chondrocytes are also seen in the mutant costal cartilage (Fig. 5I). In addition, overgrowth of the other hyaline cartilage including the trachea (Fig. 5C–E) and the nasal septa (Fig. 5L–O) is seen. Interestingly, the collagen-staining revealed a thinner layer of perichondrium in the mutant costal cartilage (Fig. 5K).

Abnormal chondrocyte proliferation in the mutant mice

In order to see if the K644M mutation causes increased chondrocyte proliferation, in vivo [3H-methyl]-thymidine incorporation experiments were performed in the SADDAN mouse embryos. The proliferating cells were found scattered in chondrocytes in both proliferating and resting zones of the growth plates and also throughout the costal cartilage (data not shown). The proliferation index in the mutant growth plate chondrocytes was 124% of the wild-type at embryonic day (E) 15.5 and the difference became higher at E18.5 (164%). In the costal cartilage, the difference was 138% at E15.5 and was higher at E18.5 (227%). The dwarf mouse models with the Fgfr3 mutations in the previous reports showed inhibition of growth plate chondrocyte proliferation in the postnatal stage (11,19–21). To determine if the SADDAN mice also show inhibition of chondrocyte proliferation in the postnatal stage, we performed immuno-histochemistry using an S phase marker, proliferating-cell nuclear antigen (PCNA) (Fig. 4M and N). The proliferating cells reactive with the PCNA antibody are found limited to the proliferating and maturating zones of the P17 growth plates. Consistent with the previous reports on the postnatal-onset dwarf mice with activating Fgfr3 mutations, the SADDAN mutant mice show decreased proliferation in the postnatal growth plate chondrocytes.

Characterization of the Fgfr3 proteins in chondrocytes isolated from the mutant mice

To see whether the constitutive activation of Fgfr3 mutant proteins could be confirmed in vivo, we analyzed the Fgfr3 proteins in chondrocytes isolated from the SADDAN and TDII mutant mice, using their wild-type littermates as controls. Expression levels of Fgfr3 are similar in TDII and SADDAN...
mutant mice (Fig. 6A), excluding the possibility that the stability or the amount of Fgfr3 is causing the phenotypic differences seen in these disorders. Even though the phosphorylation of cellular proteins was not as dramatically high as seen in in vitro studies, somewhat higher phosphorylation was indeed observed in some of the proteins in the SADDAN mutant lysates compared to that of TDII (Fig. 6B). We also examined the phosphorylation of Map kinase, since its activation was previously reported in the cells expressing the mutant FGFR3 (22). However, no significant difference was observed under the conditions used here (data not shown). Next, we tested the response of cells upon FGF treatment. To examine the consequence of the activation of Fgfr3 proteins, we used the phosphorylation states of Map kinase as an indicator.

Figure 4. Decreased differentiation of the long bones of the SADDAN mice. The growth plates of P1 tibiae of wild-type (A and B) and the SADDAN mutant mice (C and D). The enlarged views of the areas enclosed in the boxes are shown in (B and D). The total length of the long bone is shorter in the mutant mice (narrow bars), particularly prominent in the ossified zones (thick bars). The mutant growth plate chondrocytes have shorter columnar structures of the proliferating zone and some of the undifferentiated chondrocytes intermingle with the hypertrophic chondrocytes (arrow in D). At E15.5, femurs of wild-type (E) and the SADDAN mutant mice (F) show that the chondrocytes in the medial part are smaller and premature in the mutant (arrowheads). The longitudinal formation of the bone collar is similar between the wild-type and mutant mice; however, the thickness of the bone was increased (arrows). The growth plates of P4 femurs of wild-type (G) and the SADDAN mutant mice (H) show that the hypertrophic chondrocytes are sparse and not fully mature in the mutant. However, the penetration of the blood vessels from the articular surface is observed at this stage similar to the wild-type (arrows). The chondrocytes around the blood vessel in epiphysis are smaller in the mutant mice (arrowheads). The P17 femur growth plates of wild-type (I and K) and the SADDAN mice (J and L). The enlarged views of the areas enclosed in the boxes are shown in (K and L). The mutant physis (J and L) show ingrowth of mesenchymal tissue. Consistent with the younger mice, the thicker growth plates with shorter hypertrophic zones and proliferating columns are observed in the mutant. The wild-type (wt) and the SADDAN mutant (+/K644M) bone sections are stained with hematoxylin and eosin (H&E). (M and N) Immunohistochemistry with the PCNA antibody at P17 femur growth plate shows fewer proliferating cells (stained in brown) in the mutant. The zones of proliferation (p), prehypertrophic (ph), and hypertrophic (h) chondrocytes are indicated in (B and K). Scale bar, 640 µm in (I, J); 260 µm in (A, C, E–H); 130 µm in (B, D, K, L); 100 µm in (M and N).
One ng/ml of FGF1 and 10 ng/ml FGF9 shows increased Map kinase phosphorylation in the SADDAN mutant chondrocytes while a higher concentration is needed for the wild-type (Fig. 6C). The TDII chondrocytes do not show any obvious difference to the wild-type chondrocytes in the dose response experiment (Fig. 6C and data not shown). In addition, the time course of the Map kinase activation in the presence of a high FGF1 concentration (100 ng/ml) is similar in wild-type, SADDAN and TDII mutant cells (Fig. 6D). However, at the lower concentration (1 ng/ml), the highest activation was observed at 5 min in SADDAN cells, while maximum phosphorylation in the wild-type littermate cells was observed at 8 h. In the TDII mutant cells, both wild-type and mutant chondrocytes show the highest activation at 5 min at the low FGF1 concentration.

**DISCUSSION**

Detailed analyses of the SADDAN phenotype in humans are difficult due to the limited case numbers, as well as the limited accessibility of human tissues. In this study, we analyzed the long bones and cartilages of SADDAN mutant mice and
compared them with those of the TDII model. The morphologic and histologic features of human SADDAN are well represented in the SADDAN mice (Fig. 4). In humans, skeletal abnormalities in SADDAN are reported to be always more severe than ACH or TDII and as severe as in TDI (13,14). In contrast, the overall skeletal phenotype is milder in SADDAN mice compared with TDII mice. TDII mice also show curved tibia and fibula, whereas the SADDAN mice show rather straight long bones at P1 (Fig. 3C). Besides the limited numbers of SADDAN cases, the species differences of humans and mice may account for these observations on long bones characteristics. Furthermore, human SADDAN studies (13,14) reported that one of the four patients died 6 h after birth from respiratory failure, while three other patients survived till adulthood. Whether the difference in the survival of these patients (all with the same K650M mutations) corresponds to that of the two mutant SADDAN mice differing in severity is unclear and awaits more clinical cases. Finally, no obvious acanthosis nigricans or seizures were observed in the SADDAN mice. Detailed analyses of these features specific to SADDAN will require future studies.

Figure 6. Characterization of the Fgfr3 proteins in chondrocytes isolated from the SADDAN and TDII mutant mice. (A) The whole-cell lysates from SADDAN (M), TDII (E) and Fgfr3 knockout mouse (−/−) littermates are examined by a blot with anti-Fgfr3 antibody. Comparison with the Fgfr3 knockout mice sample identifies the 150 kDa band as the Fgfr3 protein. Levels of Fgfr3 proteins are similar in wild-type and mutant mice, in both SADDAN and TDII mouse models. (B) Phosphorylation of the cellular proteins is slightly higher in the SADDAN mutant than in TDII in a blot with anti-phosphotyrosine antibody, 4G10. The differences between wild-type and mutant TDII chondrocytes are not significant. The two strongly phosphorylated bands (~150 and 100 kDa) are not Fgfr3 proteins based on comparisons of their molecular weights. (C) Dose response of the cellular response to FGF ligands. The chondrocytes were plated out at a high-density (22) and treated with increasing amounts of FGF1 or FGF9 for 5 min. The whole-cell lysates were blotted with phosphorylated Map kinase (pMapK) and Map kinase (MapK) antibodies. (D) Time course of cellular response to the FGF treatments. Cells were incubated with FGF1 for 0 and 5 min, 8, 24, 48, and 72 h. Experiments were repeated at least three times using different batches of littermates and a representative blot is shown.
observed throughout development in SADDAN mutant mice, including early embryonic (E15) to postnatal (P17) growth plates (Fig. 4). Interestingly, embryonic growth plates in both SADDAN and TDII mice show increased chondrocyte proliferation compared with those in the wild-type mice (Table 1). This increase in chondrocyte proliferation was revealed to be transient and the peak of their effect was reached early (E15) in the TDII mice (15) and later (E18) in the SADDAN model. Consistent with the previous dwarf mouse models, the SADDAN mice show inhibition of the chondrocyte proliferation at the postnatal stage (P17) (Fig. 4M and N). A previous study has indicated that a slowed differentiation may lead to an accumulation of thymidine-labeled cells over time (19). The increased difference in proliferation index from E15.5 to E18.5 in SADDAN mice may be due to the accumulation of the [3H-methyl]-thymidine positive cells in the growth plates resulting from the decreased differentiation. It is not clear if the other postnatal-onset dwarf models show increased chondrocyte proliferation in the embryonic stages. It would be interesting to investigate how the strong activation of Fgfr3 stimulates chondrocyte proliferation in the embryonic stages and inhibits it postnatally. The chondrocyte overproliferation may also enhance the decrease in their differentiation, which could cause the more severe postnatal phenotype of SADDAN mice compared with the other postnatal-onset dwarf models (11,19–21).

The unexpected finding of the overgrowth of costal cartilage, trachea and nasal septum in the SADDAN mice uncovers the function of Fgfr3 in the hyaline cartilage in addition to the long bone growth plates. The hyaline cartilage in these tissues does not necessarily become ossified. Perichondrium is essential for the growth and maintenance of cartilage. The inner layer of the perichondrium contains chondroblasts that differentiate into chondrocytes. The reduction of the collagen-stain positive (blue) layer in the SADDAN costal cartilage (Fig. 5J and K) may mean decreased potential for the differentiation into the mature hyaline cartilage. We speculate that this would cause accumulation of premature chondrocytes that proliferate at a higher rate than mature chondrocytes. Further studies on the expression of Fgfr3 and the related markers would be necessary to fully understand this observation.

The discrepancy of function–phenotype correlation between TDII and SADDAN is still unsolved, i.e. the higher activation of Fgfr3 with K644M mutation with a milder cartilage phenotype. A study using a chimeric construct with the FGFR3 intracellular domain showed that the K650M and K650E mutants were expressed as two bands: a low molecular weight protein (p130) which exhibits strong ligand-independent tyrosine phosphorylation, and a fully glycosylated protein p170, which exhibits ligand-dependent activation (23). The 150 kDa Fgfr3 protein observed in the native chondrocytes (Fig. 6) is most likely a fully glycosylated form, suggesting the dominant population of Fgfr3 protein in vivo to be the ligand-responsive form on the cell surface. The biochemical study using the human primary chondrocytes from patients with the TDII mutations (located in the ligand-binding and transmembrane domains) has shown that the Map kinase activation was achieved at 2 min in the TDII chondrocytes, as compared with 5 min in controls upon FGF9 stimulation (22). Our data are consistent with this observation. The current study further demonstrates that the concentration of ligands could influence the cellular phosphorylation (measured by Map kinase phosphorylation) by Fgfr3 mutant proteins, in particular, when the lower dosage of stimulation is used. However, further studies are still necessary to clarify the discrepancy of function–phenotype correlation of TDII and SADDAN. It is possible that the other known targets of FGF receptors, including Stat, PLCγ and PI3K (3), may be involved in the differential regulation of signaling downstream of the mutant Fgfr3 proteins. We plan to proceed further with the functional analysis of the Fgfr3 mutant proteins from the in vivo source to see if there is any difference from the observations in the in vitro expression studies. The higher ratio of auto-phosphorylation in the K644M mutant protein by pY20 blot than by γ-ATP incorporation assay (Fig. 1) suggests the involvement of the abnormal phosphorylation of additional tyrosine residues. There are 12 tyrosines in the intracellular domain of FGFR3 which are conserved among FGFR1–3. Seven tyrosines are autophosphorylated in FGFR1 (24,25), five of which are also conserved in FGFR3. The identities of

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### Table 1. Enhanced proliferation of chondrocytes in the SADDAN mutant embryos

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Embryonic day (E)</th>
<th>Genotype</th>
<th>No. of sections (n)</th>
<th>Proliferation indexa</th>
<th>Proliferation indexb</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
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<td>Growth plates</td>
<td>E15.5</td>
<td>Wild-type</td>
<td>8 (n = 24)</td>
<td>0.142 ± 0.048</td>
<td>124</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
<td>+/K644E</td>
<td>Wild-type</td>
<td>8 (n = 24)</td>
<td>0.176 ± 0.054</td>
<td>124</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td></td>
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<td>164</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Costal cartilage</td>
<td>E15.5</td>
<td>Wild-type</td>
<td>4 (n = 12)</td>
<td>0.159 ± 0.030</td>
<td>138</td>
<td>&lt;0.05</td>
</tr>
<tr>
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<td>0.0736 ± 0.028</td>
<td>227</td>
<td>&lt;0.05</td>
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aProliferation index was calculated as a ratio of proliferating cells which had incorporated radiolabeled thymidine to the total cell number present in each of three given areas of the sections (total number, n) of distal femurs or proximal tibia, randomly chosen in resting and proliferating zones (growth plates), or in costal cartilage.
the cellular substrates phosphorylated by the Fgfr3 mutant proteins are not assessed in the current report and await future studies. The comparison of the abnormal activation of K650M and K650E mutant proteins is an effective approach for the understanding of the regulatory mechanism of chondrocyte proliferation and differentiation by FGFR3.

In summary, we have generated a novel mouse model with Fgfr3-K644M mutation which shows characteristics similar to human SADDAN. Together with the TDII model, it will be an important tool to study the disease mechanisms of severe FGFR3-related skeletal disorders. In particular, comparisons of these two models will give important insights into critical factors that determine the perinatal survival of the two syndromes.

MATERIALS AND METHODS

Construction of the in vitro expression vectors

The coding sequence with 18 bp of 5′-UTR of the mouse Fgfr3 IIIc cDNA (3.7 kb in total, SacII to XbaI site of MoFR3/sv; Dr D. Ornitz) was inserted into the pcDNA3.1zeo* (Invitrogen). The mutations were generated by PCR using primers, 5′-GGCTCGAGATGTGCACAACCT- GGACTACTACAAGAAG-3′ and 5′-GGCTCGAGATGTGCACAACCT-GGACTACTACAAGAAG-3′ (underlined AAG was mutated to GAG, ATG and AAT, to give E, M and N mutant receptors, respectively) and 5′-CAGACTACAAGAAGACCCAC-3′. The PCR products were digested with XhoI and BglII, and ligated into the EcoRI-XbaI 3 kb fragment, then into the pcDNA3.1 which contained the 5′ SacII-EcoRI portion.

In vitro expression, immunoprecipitation, the kinase assay and immunoblot

293 cells (American Type Culture Collection) were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine and penicillin-streptomycin at 37°C in 5% CO₂. 1 × 10⁶ cells/well were plated in 6-well plates. Cells were transfected with 1 μg of vector DNAs using lipofectamine plus Tel: +1 301 401 7225; Fax: +1 301 480 1135; Email: chuxiad@bdg10.niddk.nih.gov

REFERENCES


was performed using NIH Image (http://rsb.info.nih.gov/nih-image/).

Generation of the SADDAN mice, phenotype analyses and thymidine incorporation

Site-directed mutagenesis and the construction of the targeting vector were performed exactly as described by Iwata et al. (15) with the exception of the PCR primer F2122M (5′-GGCTCGAGATGTGCACAACCT-GGACTACTACAAGAAG-3′) instead of F2122E. The mouse line was generated and genotyped in the same fashion as we generated the TDII (15). Four chimera lines (129 strain) were obtained out of 169 ES clones resistant to G418/Flau double selection. Agouti male chimeras were mated with NIH Black Swiss females (Taconic). The resulting ‘carrier’ (Fgfr3+/K644Mneo) was mated with Ella-Cre mice (FVB/N), which ubiquitously expresses the Cre-recombinase starting from one-cell zygote stage (26). Whole skeleton staining, histologic sections and stains were performed as described or according to the standard procedure. Chondrocyte proliferation was assayed in vivo by intraperitoneal injections of pregnant mice with methyl-3H-thymidine (Amersham-Pharmaccia, 79 Ci/μmol (20 μl/mg body weight, 4 h). Immunohistochemistry with anti-PCNA antibody (Signet) was performed using standard procedures.

Primary chondrocyte culture

Sterni and costal cartilage were dissected from the pups at P1. The tissues were cut into small pieces and incubated in 2 mg/ml collagenase II (Life Technologies) in PBS for 3 h (27). Dissociated cells were washed twice with PBS and added to 50 μl of the loading buffer (the whole-cell lysate). For primary culture, cells were counted and 2–3 × 10⁵ cells/well were plated in 24-well plates in DMEM with 10% FBS. After depletion in serum-free media for 24 h, cells were incubated in the serum-free media containing 10 μg/ml heparin (Sigma) and FGF1 or FGF9 (R&D Systems) at the concentrations indicated.

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Human Molecular Genetics, 2001, Vol. 10, No. 12 1263


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