Thanatophoric dysplasia (TD) is a lethal skeletal disorder caused by recurrent mutations in the fibroblast growth factor receptor 3 (FGFR 3) gene. The mitogenic response of fetal TD I chondrocytes in primary cultures upon stimulation by either FGF 2 or FGF 9 did not significantly differ from controls. Although the levels of FGFR 3 mRNAs in cultured TD chondrocytes were similar to controls, an abundant immunoreactive material was observed at the perinuclear level using an anti-FGFR 3 antibody in TD cells. Transduction signaling via the mitogen-activated protein kinase pathway was assessed by measuring extracellular signal-regulated kinase activity (ERK 1 and ERK 2). Early ERKs activation following FGF 9 supplementation was observed in TD chondrocytes (2 min) as compared with controls (5 min) but no signal was detected in the absence of ligand. By contrast ligand-independent activation of the STAT signaling pathway was demonstrated in cultured TD cells and confirmed by immunodetection of Stat 1 in the nuclei of hypertrophic TD chondrocytes. Moreover, the presence of an increased number of apoptotic chondrocytes in TD fetuses was associated with a higher expression of Bax and the simultaneous decrease of Bcl-2 levels. Taken together, these results indicate that FGFR 3 mutations in TD I fetuses do not hamper chondrocyte proliferation but rather alter their differentiation by triggering premature apoptosis through activation of the STAT signaling pathway.

Fibroblast growth factor receptor 3 (FGFR 3) belongs to a class of tyrosine kinase receptors involved in signal transduction. In the presence of soluble or cell-surface heparan sulfate proteoglycans, fibroblast growth factor (FGF) binding to FGFRs induces receptor dimerization and autophosphorylation on tyrosine residues thus triggering cell proliferation or differentiation through the Ras-Raf-dependent and phospholipase Cγ-dependent signal transduction pathways involving MAPK stimulation (2–4).

FGFR 3 mutations have been recently shown to account for achondroplasia (5, 6), hypochondroplasia (7), and thanatophoric dysplasia (TD I and TD II) (8, 9). Based on expansion of the cartilage growth plate in Fgfr 3 null mice, FGFR 3 was regarded as a negative regulator of long bone growth during endochondral ossification (10, 11). Subsequent transfection experiments in immortalized cell lines have shown that FGFR 3 mutations trigger constitutive activation of the receptor in a ligand-independent manner (12–14). Activation of the receptor would result in the recruitment of two different Grb2-Sos adapter complexes leading to activation of the Ras-MAPK signaling pathway (4), but activation of the STAT pathway via the nuclear translocation of Stat 1 has been also demonstrated in chondrocytes of TD II patients (15). Yet, the mechanisms leading to disorganization of the growth plate cartilage in TD remains unclear and the question of whether TD mutations interfere with chondrocyte proliferation or alter terminal differentiation is still open. In an attempt to address this issue, chondrocytes isolated from cartilage of TD I fetuses were grown in primary cultures and used for proliferation assays, transduction signaling, and programmed cell death analyses. The normal mitogenic response of TD chondrocytes along with the high figure of apoptotic cells in TD fetuses suggest that FGFR 3 mutations alter differentiation rather than proliferation by inducing premature apoptosis of chondrocytes.

MATERIALS AND METHODS

Cartilage Samples—Thibial and/or femoral cartilage fragments were obtained from medically aborted TD fetuses following the informed consent of parents. In all cases, pregnancy was legally terminated after ultrasonographic and x-ray detection of lethal dwarfism. Histological studies of cartilage sections supported the diagnosis of TD I. The control group included spontaneously aborted fetuses showing no evidence of skeletal abnormalities.

Antibodies and Ligands—Rabbit polyclonal anti-FGFR 3, anti-FGFR 1, anti-Bax, and mouse monoclonal anti-PCNA antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and a polyclonal anti-FGFR 3 antibody was kindly provided by Dr. A. Yayon (Rehovot, Israel). A mouse monoclonal anti-Bcl-2 antibody was purchased from Dako (Glostrup, Denmark). A rabbit polyclonal anti-MAPK antibody was purchased from Sigma and the anti-active MAPK polyclonal antibody raised against the dually phosphorylated region of MAPK was from Promega. An anti-lysosome-associated membrane protein monoclonal antibody was obtained from Pharmigen (San Diego, CA).

Monoclonal antibodies against the C-terminal domain of human Stat 1 and against nucleoprin p62 were purchased from Transduction Laboratory. The anti-mouse phosho-Stat-1 rabbit polyclonal antibody was from Upstate Biotechnology.

The MAPKK/MEK-1 inhibitor, PD 98059, originated from New England Biolabs (Beverly, MA). A monoclonal anti-β-tubulin antibody origi-
inated from Amersham. The anti-digoxigenin-AP, the substrate nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, and the in situ cell death detection kit were purchased from Boehringer Mannheim. Human FGF 2, EGF, and mouse FGF 9 were purchased from Preprotech (Rocky Hill, NJ).

DNA Analyses—Screening of FGF 3 mutations on either white blood cell or cultured skin fibroblast DNA was performed by single strand conformation polymorphism and restriction analyses or by direct sequencing of amplification products as described (16).

Chondrocyte Cultures and Proliferation Studies—Cartilage fragments were dissected free of perichondrium and cut into small slices. Chondrocytes were released by hyaluronidase-trypsin digestion followed by collagenase treatment (17). Primary cultures were initiated by plating cells at high density to maintain their differentiated phenotype (0.8–1.2 × 10^5 cells/cm²). For proliferation studies, freshly isolated chondrocytes were plated on 96-well dishes (Falcon) in DMEM, supplemented with 10% fetal calf serum and antibiotics and were allowed to reach subconfluence. Cells were rinsed three times with serum-free DMEM containing 25 mM Hepes, pH 7.4, and incubated in the same medium at 37 °C for 16 h (or for times varying from 4 to 24 h) in the presence of increasing concentrations of either FGF 2 or FGF 9 (10–100 ng/ml) at a final heparin concentration of 10 μg/ml. Nonstimulated cells were incubated in serum-free DMEM with or without heparin. In some experiments early confluent cells were synchronized by exposure to DMEM containing 1% fetal calf serum and antibiotics and were allowed to attach for 4–5 days. Cells were rinsed twice with PBS, fixed in 4% paraformaldehyde in PBS for 30 min, and incubated for 30 min at 37 °C in the presence of 800 units/ml hyaluronidase (Sigma). Permeabilization with Triton X-100 (0.1%) was followed by incubation with the appropriate primary antibody for 1 h at room temperature. After washing three times with PBS-gelatin (0.2%) and Triton X-100 (0.1%), the rabbit fluorescein isothiocyanate-labeled or mouse Cy3-conjugated secondary antibody was added and the mixture was incubated for 1 h in a dark room. Then cells were covered with mounting solution and examined with a Leica microscope equipped for fluorescence.

Northern Blot and In Situ Hybridization Studies—Total RNA extraction from cultured chondrocytes, formaldehyde gel electrophoresis, and blotting onto Hybond N membranes were performed as described previously (17). Probes were labeled by random priming with [α-32P]dCTP and used at 10–50 ng/ml for 24 h at 42°C. For in situ hybridization, sense and antisense riboprobes were synthesized using either the Sp6 or the T3-T7 RNA polymerases in the presence of dUTP-digoxygenin (Boehringer Mannheim). Cells were then washed twice with PBS and incubated for 4 h in serum-free DMEM supplemented with [3H]thymidine (ICN) at a concentration of 10 μCi/ml (specific activity: 6.7 Ci/mmol) in a final volume of 100 μl. Cells were harvested on a glass fiber filter paper and assayed for radioactivity by liquid scintillation counting. Cells cultured under identical conditions but in the absence of [3H]thymidine were used to evaluate the number of cells per well.

Immunofluorescence Analyses—Chondrocytes were plated on tissue culture chamber slides (Nunc Inc.) at a density of 20,000 cells/chamber in DMEM (200 μl) supplemented with 10% fetal calf serum, and allowed to attach for 3–5 days. Cells were rinsed twice with PBS, fixed in 4% paraformaldehyde in PBS for 30 min, and incubated for 30 min at 37 °C in the presence of 800 units/ml hyaluronidase (Sigma). Permeabilization with Triton X-100 (0.1%) was followed by incubation with the appropriate primary antibody for 1 h at room temperature. After washing three times with PBS-gelatin (0.2%) and Triton X-100 (0.1%), the rabbit fluorescein isothiocyanate-labeled or mouse Cy3-conjugated secondary antibody was added and the mixture was incubated for 1 h in a dark room. Then cells were covered with mounting solution and examined with a Leica microscope equipped for fluorescence.

RESULTS

FGFR 3 Mutations—Single strand conformation polymorphism and restriction analyses of the coding sequence of the FGF 3 gene led to the detection of deleterious mutations in 14/14 TD I fetuses. Mutant genotypes included the R248C (8/14), S249C (2/14), J807G (1/14), and Y373C (3/14) mutations (Table I).

Phenotypic and Genotypic Characterization of Human Cultured Chondrocytes—When plated at a density of 8 × 10⁴ cells/cm², control and TD chondrocytes reached subconfluency in 5 days. In situ hybridization of cultured chondrocytes with an antisense COL2A1 riboprobe gave a strong signal in most control and TD cells (Fig. 1, a and b), demonstrating that growing normal or TD chondrocytes at a high density on plastic substrate did not result in a rapid cell dedifferentiation to a mesenchymal state. Consistently, Northern blot analysis of chondrocyte mRNAs with α1(I) cDNA probe showed high levels of collagen type I gene expression in both control and TD chondrocytes (Fig. 2). Subsequent hybridization of TD cells with an antisense FGF 3 riboprobe revealed a faint signal which matched that found in control chondrocytes (Fig. 1, c and d). Northern blot analysis revealed low levels of FGF 3 IIIc mRNAs and high levels of FGF 1 mRNA transcripts in both control and TD chondrocytes (Fig. 2), while FGF 3 IIIb and FGF 2 mRNAs were undetectable (not shown).

Localization of FGF 3 Proteins in TD Chondrocytes—The subcellular localization of FGF 3 proteins in cultured chondrocytes was investigated by using an antibody raised against the C-terminal end of the protein. In control cells, immunofluorescence was mostly visible in the cytoplasm and a faint staining was occasionally seen around the nucleus. Surprisingly, immunofluorescence was mostly visible in the cytoplasm and a faint staining was occasionally seen around the nucleus. Surprisingly, counterstaining of nuclei with 4',6-diamidino-2-phenylindole dihydrochloride revealed a strong perinuclear staining in 40–50% of TD I chondrocytes regardless of the FGF 3 mutations (Fig. 3, d-f). This distribution was not altered by a overnight serum deprivation or by FGF 2 or FGF 9 stimulation (5–30 min). In an attempt to confirm the perinuclear localization of

lipore Corp) were performed according to standard procedures, and blots were sequentially hybridized overnight at 4 °C in 5% nonfat milk and 0.1% Tween 20 with anti-MAPK polyclonal antibody and MAPK antibodies at a 1:5,000 final concentration. A second antibody coupled to peroxidase was added. Bound protein was detected by chemiluminescence (ECL, Amersham). For analysis of the STAT pathway, nylon membranes were sequentially hybridized overnight at 4 °C with anti-phospho Stat 1 and anti-Stat 1 antibodies at respective dilutions of 1:1,000 and 1:5,000.

Analysis of Apoptotic Cells and Apoptotic Factors—Immunohistochemical detection of apoptosis in primary cultured chondrocytes was achieved according to the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay using an in situ cell death detection kit (Boehringer Mannheim). Briefly, fragmented DNA and apoptotic bodies were labeled with fluorescein-12-dUTP using terminal deoxynucleotidyl transferase. Incorporated fluorescein was visualized by incubation with an anti-fluorescein antibody conjugated with alkaline phosphatase. After chromogen substrate reaction, apoptotic and non-apoptotic cells were counted under a light microscope. For detection of anti- and pro-apoptotic factors, Western blots were hybridized with anti-Bcl-2 and anti-Bax antibodies at respective dilutions of 1:200 and 1:500. The blots were rehybridized with an anti-β-tubulin antibody for quantitation (1:10,000 dilution).

Immunohistochemical Techniques—Tibial cartilage fragments from TD and control chondrocytes were fixed with 4% paraformaldehyde and embedded in paraffin. Serial sections were stained with hematoxylin-eosin or pretreated with hyaluronidase (800 units/ml) for 30 min at 37 °C, then incubated for 1 h with an antibody against PCNA (a 36-kDa auxiliary protein of DNA polymerase α) at a 1:50 dilution for assessment of cells undergoing proliferation. Other paraffin sections were incubated for 1 h with an anti-Stat 1 monoclonal antibody (1:100 dilution). Sections were then reacted with horseradish peroxidase-conjugated secondary antibody and dianisobenzidine was used as a substrate for visualization (Boehringer Mannheim).

A clear reduction in staining intensity of collagen type II gene expression in both control and TD chondrocytes (Fig. 2), while FGFR 3 IIIb and IIIc FGFR 2 mRNAs were undetectable (not shown).

Evaluation of the phenotype and genotype of human cultured chondrocytes showed that expression of FGF 3 was reduced in TD chondrocytes (Fig. 2). This reduction was not due to a decrease in the expression of FGFR 3 proteins, as evaluated by immunofluorescence and Western blotting (Fig. 3, d-f).

Localization of FGF 3 Proteins in TD Chondrocytes—The subcellular localization of FGF 3 proteins in cultured chondrocytes was investigated by using an antibody raised against the C-terminal end of the protein. In control cells, immunofluorescence was mostly visible in the cytoplasm and a faint staining was occasionally seen around the nucleus. Surprisingly, counterstaining of nuclei with 4',6-diamidino-2-phenylindole dihydrochloride revealed a strong perinuclear staining in 40–50% of TD I chondrocytes regardless of the FGF 3 mutations (Fig. 3, d-f). This distribution was not altered by a overnight serum deprivation or by FGF 2 or FGF 9 stimulation (5–30 min). In an attempt to confirm the perinuclear localization of
FGFR 3 in TD cells, double immunostaining experiments were performed with both anti-FGFR 3 (green) and anti-nucleoporin antibodies (red). Co-localization of the receptor with nucleoporin, a marker of the nuclear membrane, was demonstrated by the appearance of an intermediate color (yellow) around the nucleus (Fig. 3, g-i). By contrast, double immunostaining with antibodies against FGFR 3 and lysosome-associated membrane protein 2 (a lysosomal marker), only revealed a partial overlap of the signals suggesting that mutant FGFR 3 was not preferentially retained in lysosomes for degradation (Fig. 3, j-l). Finally, staining with an antibody directed against the mannose 6-phosphate receptor, a specific marker of the late endosomes showed no evidence for co-localization of the two receptors (not shown). Bars = 20 μm.

FGFR 3 in TD cells, double immunostaining experiments were performed with both anti-FGFR 3 (green) and anti-nucleoporin antibodies (red). Co-localization of the receptor with nucleoporin, a marker of the nuclear membrane, was demonstrated by the appearance of an intermediate color (yellow) around the nucleus (Fig. 3, g-i). By contrast, double immunostaining with antibodies against FGFR 3 and lysosome-associated membrane protein 2 (a lysosomal marker), only revealed a partial overlap of the signals suggesting that mutant FGFR 3 was not preferentially retained in lysosomes for degradation (Fig. 3, j-l). Finally, staining with an antibody directed against the mannose 6-phosphate receptor, a specific marker of the late endosomes showed no evidence for co-localization of the two receptors (not shown). Bars = 20 μm.

Mitogenic Response of TD Chondrocytes—Our observation that cultured chondrocytes expressed both FGFR 1 and FGFR 3 suggested that either FGF 2 or FGF 9 could be used to measure cell proliferation, especially as they efficiently bind FGFR 1 and FGFR 3 IIIc, respectively (20–22). Consequently, subconfluent control chondrocytes were first incubated in the presence of FGF 9. A peak of thymidine incorporation was noted after a 16–24-h FGF 9 stimulation (Fig. 4a). In subsequent proliferation studies, FGF-supplemented cells were incubated for 16 h prior to thymidine uptake measurement. Then we analyzed the influence of synchronization on the proliferative activity of control and TD chondrocytes. Cells were serum-deprived for 24 or 48 h before supplementation of FGF 9 ligand. While FGF 9 had a significant mitogenic effect on non-deprived cells, serum depletion for 24 or 48 h resulted in a marked reduction of thymidine incorporation (Fig. 4b). In the presence of heparin, cells exhibited a dose-dependent proliferative response which revealed a maximum stimulation for FGF 2 or FGF 9 concentrations ranging from 50 to 100 ng/ml (Fig. 4c). The proliferation of chondrocytes from TD I fetuses harboring various FGFR 3 mutations was compared with age-matched control cells. TD chondrocytes showed a magnitude of stimula-

### Table I

Summary of FGFR 3 mutations and analytical methods used on primary cultured TD chondrocytes and cartilage samples

Twenty age-matched control fetuses (gestational age ranging from 16 to 30 weeks) with no clinical or radiological signs of skeletal defects were used for comparison.

| Patient | Age | Skeletal disorder | FGFR3 mutations | Signal transduction (MAPK/Stat1) | IF | Proliferation FGF2/FGF9 | Apoptosis | Immunohistochemistry |
|---------|-----|------------------|----------------|---------------------------------|----|--------------------------|----------|----------------------|
| 1       | 27  | TDI              | R248C          | Y                               | Y  | Y                       | Y        | Y                    |
| 2       | 26  | TDI              | R248C          | Y                               | Y  | Y                       | Y        | Y                    |
| 3       | 19  | TDI              | R248C          | Y                               | Y  | N                       | Y        | Y                    |
| 4       | 34  | TDI              | R248C          | Y                               | Y  | N                       | Y        | Y                    |
| 5       | 22  | TDI              | S249C          | Y                               | Y  | N                       | Y        | Y                    |
| 6       | 25  | TDI              | R248C          | Y                               | Y  | N                       | Y        | N                    |
| 7       | 23  | TDI              | S249C          | Y                               | Y  | Y                       | N        | N                    |
| 8       | 38  | TDI              | R248C          | Y                               | Y  | N                       | N        | N                    |
| 9       | 16  | TDI              | J807G          | N                               | N  | Y                       | N        | Y                    |
| 10      | 21  | TDI              | Y373C          | Y                               | Y  | N                       | N        | N                    |
| 11      | 24  | TDI              | Y373C          | Y                               | Y  | Y                       | N        | N                    |
| 12      | 25  | TDI              | R248C          | Y                               | Y  | Y                       | N        | N                    |
| 13      | 23  | TDI              | Y373C          | Y                               | Y  | Y                       | N        | N                    |
| 14      | 17  | TDI              | R248C          | Y                               | Y  | Y                       | N        | N                    |
tion by FGF 2 and FGF 9 that did not significantly differ from that of controls (Fig. 4d). Binding experiments with \( ^{125}\text{I}-\text{FGF 2} \) indicated that dissociation constants \( (K_d) \) of control or mutant cells were in the same range (1.2 \( \pm \) 0.4 \( \times \) \( 10^{-9} \) M and 0.8 \( \pm \) 0.2 \( \times \) \( 10^{-9} \) M for control and TD chondrocytes, respectively).

**Activation of the MAP Kinase Pathway in TD Chondrocytes**—Since FGFR 3 mutations are expected to produce a constitutive receptor activation and signal transduction in TD, phosphorylation of the MAPK (ERK 1 and 2) via the MAPK activation pathway was tested using an antibody against phospho-ERKs. In the absence of ligand, no phosphorylated MAPK was detected in both control and TD chondrocytes. Addition of FGF 9 to the culture medium (5–30 min) resulted in the detection of phosphorylated ERK 1 and 2 in both cell types but the intensity of the signal in TD was stronger than in control chondrocytes (Fig. 5a and b). Indeed, a short FGF 9 stimulation (100 ng/ml for 2 min) allowed to detect a specific signal in mutant but not in control chondrocytes (Fig. 5c). Although ERK phosphorylation peaked only 5 min after ligand supplementation, the specific signal was sustained for more than 2 h in TD cells (Fig. 5d). By contrast, stimulation with EGF resulted in a rapid ERK phosphorylation in both cell types which rapidly decreased after 30 min and was almost undetectable after 1 h (results not shown). Further evidence of MAPK pathway involvement upon binding of FGF 9 to FGFR 3 was given by abolition of the FGF 9-dependent MAPK phosphorylation by a MEK 1 inhibitor, PD98059, both in control and mutant chondrocytes (not shown). Finally, evidence that MAPK stimulation in TD cells was mediated by mutated FGFR 3 proteins stemmed from experiments using EGF. While adding FGF 9 to the culture medium increased MAPK phosphorylation, stimulating the EGF receptor with EGF in either control or TD chondrocytes had similar effects on the level of MAPK phosphorylation (Fig. 5e).

**Activation of the STAT Pathway in Cultured TD Chondrocytes**—The possible involvement of the STAT pathway in the signal transduction of mutant chondrocytes was tested by immunoblotting methods. Whole cell extracts were incubated with an anti-phospho-Stat 1 antibody so as to detect the activated form of the transcription factor. A significant 92-kDa
band was observed in the absence of ligand stimulation in TD cells while no detectable signal was noted in control cells (Fig. 6). FGF 9 supplementation had no effect on Stat 1 phosphorylation. Rehybridization of nylon membranes with anti-Stat 1 and anti-β-tubulin antibodies as internal standard revealed slightly higher amounts of Stat 1 in TD cells than in control cells (Fig. 6, b and c).

Immunohistochemical Analyses of TD Cartilage Growth Plates—The cartilage growth plates of control and TD I fetuses were tested using immunohistochemical methods. Staining of control cartilage with an anti-Stat 1 antibody disclosed a faint signal in the cytoplasm of hypertrophic cells while a positive staining of the nuclei was noted in TD hypertrophic chondrocytes thus confirming Stat 1 activation in TD cells (Fig. 7, c–f). Control chondrocytes in the resting and proliferative zones stained positively for PCNA, a marker of S-phase cells,
whereas normal hypertrophic cells displayed no immunoreactivity. In contrast, the nuclei of some TD chondrocytes located in the hypertrophic zone stained positively with PCNA identifying them as proliferative cells (Fig. 7, a and b).

Analysis of Apoptotic Cells—Subconfluent chondrocytes were labeled for DNA fragmentation using the TUNEL assay. Control cells from three different fetuses gave no staining while approximately 1–2% of TD cells derived from three different patients stained positively, regardless of the location of mutations (Fig. 8). Comparison of Bcl-2 and Bax levels using Western blotting indicated that Bax expression in chondrocytes derived from five TD patients was higher than in controls. On the other hand, the Bcl-2 was relatively abundant in control chondrocytes but almost undetectable in TD cells (Fig. 9). Although variations were noted between TD samples, the Bcl-2/Bax ratio was consistently reduced.

DISCUSSION

In an attempt to elucidate the functional consequences of FGFR 3 mutations on endochondral ossification, primary cultured chondrocytes from control and TD I fetuses and the growth plate of cartilage sections were studied. We first investigated the pattern of FGFR gene expression in cultured fetal chondrocytes. Northern blot and in situ hybridization experiments on normal and TD chondrocytes detected comparable levels of FGFR 3 gene expression. Similar results have been obtained with fetal cartilage sections (23). FGFR 1 was more strongly but equally expressed in both cell types, while no
FGFR 2 gene product was detected. These results are consistent with previous in situ hybridization studies which have shown that (i) the mouse Fgfr 3 gene is mainly expressed in the resting and proliferative zones of cartilage (10, 24), (ii) Fgfr 1 predominates in hypertrophic chondrocytes, and (iii) Fgfr 2 is absent from cartilage and expressed in the periosteum and perichondrium only (25, 26).

We subsequently investigated the proliferative capacities of primary cultured chondrocytes from TD I fetuses carrying the R248C and S249C mutations. Mean TD chondrocyte density at subconfluence did not significantly differ from that of age-matched controls. Similarly, mitogenic responses of control and mutant cells were in the same range, irrespective of the ligand tested. Serum deprivation equally affected the mitogenic response of both control and TD cells. Taken together, our data suggested that FGFR 3 mutations did not alter the proliferative capacities of resting chondrocytes and that the defective growth of the long bones in TD is more likely related to an abnormal cell differentiation than to a lack of chondrocyte proliferation during fetal development. These results are at variance with in vitro studies on hematopoietic BaF3 cells transfected with a mutant FGFR 3 cDNA carrying the R248C mutation. Indeed, the transfected cells failed to respond to mitogenic stimulation by FGF 1, while they actively proliferated in the absence of FGF (13). These discrepancies might be ascribed to (i) differences between the cell types and/or to (ii) homozygous for the mutation in transfected cells (while heterozygous mutations are present in TD chondrocytes).

In normal chondrocytes as in transfected cells, FGF-mediated FGFR activation induced signal transduction and triggered stimulation of the MAPK pathway (3, 4). At variance with the transient MAPK activation by EGF, FGF 9 stimulation resulted in a sustained MAPK activation, suggesting that differentiation rather than proliferation is involved (27). Studying the possible constitutive activation of the receptor in TD chondrocytes indicated that FGF 9 supplementation produced a faster phosphorylation of MAPK/ERKs in mutant cells than in controls. However, no stimulation was detected in the absence of ligand as observed in PC12 cells transfected with a chimeric mutant receptor carrying the G375C achondroplasia mutation (28). Our inability to detect ERK phosphorylation in the absence of FGF should be ascribed to (i) the low receptor number, (ii) the low kinase activity of FGFR 3 in primary cultured chondrocytes, and/or (iii) the ability of FGFR 3 to heterodimerize with FGFR 1. Alternatively, one can hypothesize that the MAPK pathway is not the only FGFR signaling pathway. A highly controlled balance between the MAPK and STAT pathways has been recently demonstrated in growth factor-stimulated cells (29). The observation of a Stat 1 activation by the recurrent K650E FGFR 3 mutation in TD II gives support to the view that this pathway might also play a key role in growth retardation (15). In keeping with this, we have observed a significant ligand-independent Stat 1 phosphorylation in cultured TD I chondrocytes and a Stat 1 nuclear staining in hypertrophic chondrocytes of TD I cartilage growth plate while a cytoplasmic staining only occurred in control cartilage cells.

Although FGFRs transfected cells have shown immunostaining patterns consistent with a plasma membrane localization of receptors (12, 30), a perinuclear localization of FGFR 1 in FGF 2-stimulated ovine epiphyseal growth plate chondrocytes or FGF 1-stimulated NIH 3T3 cells has been also reported (31, 32). Moreover, nuclear localization of FGFR 1 in FGF 2-stimulated Swiss 3T3 fibroblasts and accumulation of a truncated FGFR 3 form in the nucleus of breast epithelial cells have been demonstrated (30, 33). Thus, our detection of a perinuclear staining in cultured TD I chondrocytes with anti-FGFR 3 antibodies is not totally unexpected and seems consistent with the perinuclear localization of FGFR 3 in the cartilage growth plate of TD patients (23). If one hypothesizes that ligand-independent FGFR 3 homodimerization in TD cells mimics the ligand stimulation occurring in 3T3 cells, it is conceivable that FGFR 3 mutations triggered the translocation of the receptor from the cell surface to the juxtanuclear region (32, 34). Indeed, the co-localization of anti-FGFR 3 and anti-nucleoporin antibodies in the perinuclear compartment of TD chondrocytes gives support to this assertion and tends to exclude accumulation of the mutant receptor in the lysosome/endosome system for degradation.

The growth of tubular bones normally involves chondrocyte proliferation and differentiation into hypertrophic cells followed by replacement of cartilage by bone tissue. Indeed, terminally differentiating hypertrophic chondrocytes are known to undergo cell death through apoptosis (35, 36). The reduced length growth of long bones in TD fetuses could result from defective chondrocyte proliferation, premature terminal differentiation, or both. Our observation that chondrocytes from TD fetuses proliferated normally suggests that defective terminal differentiation of chondrocytes is involved. In keeping with this, a marked reduction in the proportion of hypertrophic
chondrocytes expressing collagen type X, a specific marker of terminal differentiation, has been observed in the growth plate of TD I patients (23) and chondrocytes expressing PCNA were found in the hypertrophic zone of TD cartilage. Interestingly, activation of the STAT signaling pathway in EGF-treated cancer cell lines has been proved to induce apoptosis (29). Hence, the Stat 1 activation in TD I hypertrophic chondrocytes along with the occurrence of apoptotic cells (1–2%) and the reduced Bcl-2/Bax ratio in TD I chondrocytes strongly suggest that apoptosis is involved in defective endochondral ossification. Indeed, programmed cell death of hypertrophic cells in normal mouse cartilage has been recently shown to be controlled by balanced levels of Bcl-2 and Bax, two members of a survival gene family with opposite effects (37). The decrease in Bcl-2 levels are expected to promote cell death, possibly through Bax homo-dimerization (38). Alternatively, stimulated expression of caspases (the ICE family of proteases, Ref. 39) through Stat 1 activation could also participate to the apoptotic pathway (15, 16). Overexpression of a dominant negative mouse FGFR 1 mutation suppressed apoptosis (41) gives support to the view that FGFR mutations producing a gain of receptor function increase apoptosis.

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