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Overexpression of FGFR3, Stat1, Stat5 and p21^{Cip1} correlates with phenotypic severity and defective chondrocyte differentiation in FGFR3-related chondrodysplasias

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Abstract

Achondroplasia (ACH) and thanatophoric dysplasia (TD) are human skeletal disorders of increasing severity accounted for by mutations in the fibroblast growth factor receptor 3 (FGFR3). Attempts to elucidate the molecular signaling pathways leading to these phenotypes through mouse model engineering have provided relevant information mostly in the postnatal period. The availability of a large series of human fetuses including 14 ACH and 26 TD enabled the consequences of FGFR3 mutations on endogenous receptor expression during the prenatal period to be assessed by analysis of primary cultured chondrocytes and cartilage growth plates. Overexpression and ligand-independent phosphorylation of the fully glycosylated isoform of FGFR3 were observed in ACH and TD cells. Immunohistochemical analysis of fetal growth plates showed a phenotype-related reduction of the collagen type X-positive hypertrophic zone. Abnormally high amounts of Stat1, Stat5 and p21^{Cip1} proteins were found in prehypertrophic–hypertrophic chondrocytes, the extent of overexpression being directly related to the severity of the disease. Double immunostaining procedures revealed an overlap of FGFR3 and Stat1 expression in the prehypertrophic–hypertrophic zone, suggesting that constitutive activation of the receptor accounts for Stat overexpression. By contrast, expression of Stat and p21^{Cip1} proteins in the proliferative zone differed only slightly from control cartilage and differences were restricted to the last arrays of proliferative cells. Our results indicate that FGFR3 mutations in the prenatal period upregulate FGFR3 and Stat–p21^{Cip1} expression, thus inducing premature exit of proliferative cells from the cell cycle and their differentiation into prehypertrophic chondrocytes. We conclude that defective differentiation of chondrocytes is the main cause of longitudinal bone growth retardation in FGFR3-related human chondrodysplasias.

Keywords: Cartilage; Chondrocytes; Chondrodysplasia; Fibroblast growth factor receptor (FGFR); STAT signaling

Introduction

Fibroblast growth factor receptors (FGFRs) constitute a family of four members with tyrosine kinase activity. These receptors and their ligands (FGFs) are involved in a variety of developmental processes. Germinal missense *FGFR3* mutations have been associated with various human inherited skeletal disorders including chondrodysplasias and craniosynostoses, while *FGFR1* and *FGFR2* mutations only account for craniosynostoses [4,21,42]. More recently, some forms of cancer, mostly bladder and cervix carcinomas and multiple myelomas, have been ascribed to somatic *FGFR3* mutations affecting the codons that are also involved in germinal mutations [3,5,9].

The severity of skeletal dysplasias caused by *FGFR3* mutations varies largely, ranging from mild dwarfism with normal skull (hypochondroplasia, HCH) to lethal conditions with cloverleaf skull (thanatophoric dysplasia, TD) [4,38]. While HCH and achondroplasia (ACH) are clinically homogeneous and generally accounted for by single mutations at positions 540, 650 (HCH) and 380 (ACH), respectively, thanatophoric dysplasia has been subdivided into two clinical groups (TDI and TDII) based on the aspect of the femurs (bowed in TDI and straight in TDII). TDI is caused by missense mutations creating cysteine residues in the extracellular domain of the receptor or the abolition of the translation termination codon. TDII results from a recurrent missense mutation (*K650E*) in the intracellular tyrosine kinase domain II [27,28,36]. Interestingly, conversion of

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lysine 650 into methionine (*K650M*) gives rise to TDI or a nonlethal syndrome called Severe Achondroplasia with Developmental Delay and Acanthosis Nigricans (SADDAN) [35]. This mutation and those causing TD and ACH have been demonstrated to induce constitutive activation of the receptor in transiently transfected cells [23,35,41]. While ligand-independent FGFR3 dimerization is the most likely mechanism in TDI, dimerization of the *G380R* mutant receptor apparently requires binding of a FGF ligand [20,37]. However, the downstream signaling pathways by which constitutively activated receptors exert their effects on cartilage remain unclear.

During the last 4 years, a series of mouse models aimed at reproducing human phenotypes and elucidating the signaling pathways involved in defective bone growth have been generated [7,8,15,16,18,22,31,40]. Unlike human fetuses, some of the animals harboring ACH or TD mutations had no phenotype or a mild phenotype at birth but exhibited growth retardation in the postnatal period [7,8,18,22,40]. Comparison of the signaling pathways mediating the effects of FGFR3 activation revealed some discordance between human fetuses and mouse models. Hence, during the prenatal period, expression of Indian Hedgehog (*Ihh*) and parathyroid hormone (PTH)-related peptide receptor 1 (*PTHR1*) was found unaffected in the cartilage growth plate of ACH and TD fetuses [11]. In line with this result, mouse embryos carrying the TDII mutation showed unaltered expression of *Ihh* and *Pthr1* during prenatal development [16], and FGF signaling was demonstrated to regulate chondrocyte differentiation independent of the *Ihh–Pthr1* pathway in a limb culture system [19]. By contrast during the postnatal period, *Fgfr3* was described as an upstream negative regulator of the *Ihh–Pthr1* pathway in transgenic animals overexpressing the *G380R* mutation [22], suggesting that significant differences may exist between prenatal and postnatal periods. In vitro studies have also documented some variations between human and animal cell lines. Primary cultured chondrocytes from TDI patients proliferated similarly to controls in the presence of FGF2 or FGF9 [17], whereas rat chondrosarcoma (RCS) cells cultured for long times with FGF ligands (FGF1 or FGF9) exhibited reduced proliferation and decreased FGFR3 expression when compared to unstimulated control cells [12,29,30].

These discrepancies prompted us to investigate the differentiation process in cartilage growth plates of ACH and TD fetuses. Taking advantage of a large series of human fetuses carrying *FGFR3* mutations, we show here a severity-related overexpression of FGFR3, Stat1, Stat5 and $p21^{Cip1}$ proteins in the chondrocytes of chondrodysplasic fetuses. Our results suggest that premature exit of proliferative chondrocytes from the cell cycle and accelerated differentiation into prehypertrophic chondrocytes account for the delayed longitudinal bone growth in human skeletal dysplasias caused by *FGFR3* mutations.

Materials and methods

Tissue samples

Tibial cartilage samples from ACH and TD fetuses were obtained at autopsy following ultrasound detection of intrauterine growth retardation and termination of pregnancies. In one familial case of ACH, prenatal diagnosis was performed at 12 weeks of gestation followed by termination of pregnancy at 13 weeks.

Table 1
Clinical diagnoses and FGFR3 mutations identified in fetuses reported in this study

Control Nb	Age (wks)	ACH Nb	Age (wks)	Mutation	TD II Nb	Age (wks)	Mutation	TD I Nb	Age (wks)	Mutation
1	12	1	13	G380R	1	17	K650E	1	17	R248C
2	16	2	29	G380R	2	18	K650E	2	18	Y373C
3	18	3	29.5	G380R	3	20	K650E	3	19	R248C
4	18	4	30	G380R	4	22	K650E	4	19	S249C
5	20	5	30	G380R	5	23	K650E	5	19	R248C
6	22	6	30	G380R	6	24	K650E	6	21	Y373C
7	25	7	32	G380R	7	25	K650E	7	22	S249C
8	26	8	32	G380R				8	22	R248C
9	30	9	34	G380R				9	23	R248C
10	35	10	34	G380R				10	23	S249C
		11	34	G380R				11	23	R248C
		12	35	G380R				12	24	R248C
		13	35	G380R				13	24	R248C
		14	35	G380R				14	25	R248C
								15	26	R248C
								16	26.5	R248C
								17	30	R248C
								18	37	Y373C
								19	38	R248C

Nb, number; wks, weeks.

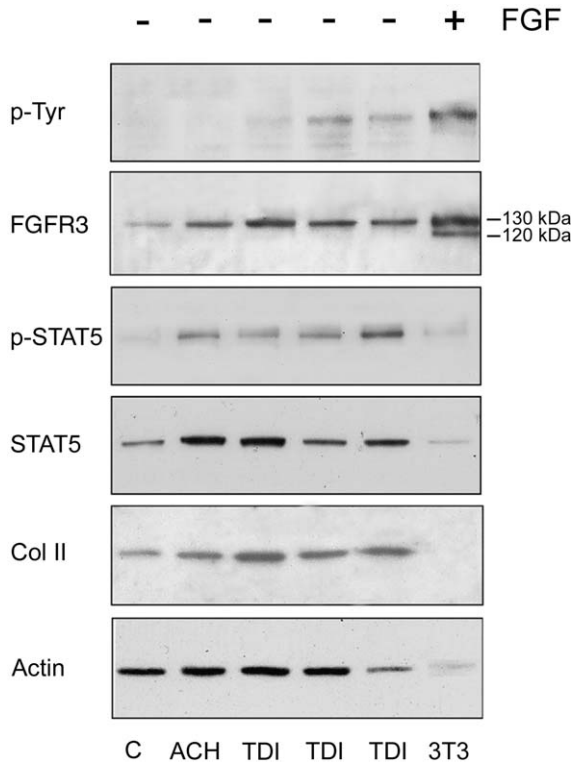

Fig. 1. Western blot analysis of primary cultured chondrocyte lysates from control (C), ACH and TDI fetuses. PVDF membranes were sequentially hybridized either with anti-phosphotyrosine (1:1000), anti-FGFR3 (1:1000) and anti-actin antibodies (1:5000) or with anti-phospho-Stat5 (1/200), anti-Stat5 (1/1000) and anti-collagen type II (1/5000) antibodies. NIH3T3 (3T3) cells stably transfected with a wild-type FGFR3 cDNA and stimulated with FGF 18 (100 ng/ml) for 5 min were used as a positive control for tyrosine phosphorylation. Two FGFR3 isoforms were detected in NIH3T3 cells but only the fully glycosylated isoform (130 kDa) was phosphorylated in the presence of FGF 18.

Control cartilage samples originated from spontaneously aborted fetuses or from medically aborted fetuses with no radiological signs of skeletal defects and no major chromosomal anomalies. In all cases informed consents of the parents were obtained and samples were processed in agreement with the French ethical committee guidelines.

Cartilage fragments were fixed for 2 h in paraformaldehyde 4% in PBS then embedded in paraffin to prepare serial sections. Histological examination was done after hematoxylin–eosin–safran (HES) staining. Samples that failed to demonstrate a satisfactory preservation of cell architecture were not used for immunostaining studies.

Mutation detection

DNA was isolated either from dermal fibroblasts or primary cultured chondrocytes then amplified by PCR using *FGFR3* primers encompassing exons 8, 10, 15 and 19. PCR conditions and primer sequences were the same as described earlier [4]. Direct sequencing of amplimers with the dideoxy termination method was performed as previously reported [28].

Chondrocyte cultures and Western blot analyses

Chondrocytes were released from cartilage fragments by sequential enzymatic digestion then plated at a high cell density in plastic flasks (10^5 cells/cm^2) to maintain their differentiated phenotype. Cells were allowed to grow for 5–6 days in DMEM supplemented with 10% FCS then used for immunochemical studies. For this purpose, cell lysates prepared from confluent primary cultured chondrocytes were submitted to polyacrylamide gel electrophoresis, electroblotted on PVDF membranes and hybridized overnight at 4°C with primary antibodies: FGFR3 (Sigma Chem.), phosphotyrosine (P-Tyr-102, Cell Signaling), phospho-Stat5a/b (Upstate Biotechnology, VA) at a 1/1000 dilution, Stat5a (Santa Cruz, CA) at a 1/200 dilution, Collagen type II (Rockland, PA) and β actin at a 1/5000 dilution. After binding of a second antibody coupled to horseradish peroxidase (HRP), proteins were visualized by chemiluminescence by ECL (Amersham).

NIH3T3 cells were stably transfected with a full-length FGFR3 cDNA kindly provided by Dr. M. Hayman (Stony Brook University, NY) using Fugene 6 (Roche). Confluent cells were depleted of serum for 16 h and incubated in the presence of FGF18 (100 ng/ml) and heparin (1 μg/ml) for 5 min, then lysed with RIPA supplemented with proteases inhibitors and analyzed by immunoblotting.

Immunohistochemical analyses

Cartilage sections were pretreated with hyaluronidase for 30 min at 37°C as described [13] then incubated with primary antibodies including FGFR3 (an antibody raised against the

Fig. 2. Histological characteristics (HES staining) and immunostaining with an anti-collagen type X antibody of tibial cartilage growth plates. No significant differences in the cellular arrangement between a 12-week-old control (A) and a 13-week-old ACH fetus (B) are visible. The size of the ACH hypertrophic zone stained with an anti-collagen type X antibody (G) looks unaffected when compared to the age-matched control (F). By contrast, the extent of chondrocyte column disorganization in 25–30-week-old ACH (D), TDII (H) and TDI (I) fetuses when compared with 26 weeks control (C) seems directly related to the severity of the disease. Immunostaining with an anti-collagen type X antibody (E, J) demonstrates the narrowing of the hypertrophic zone in TDI (J). The intensity of matrix staining in TDI sections is much lower than in control (E). pr, proliferative zone; hy, hypertrophic zone; original magnification: ×20.

Fig. 3. Immunostaining of growth plates with an anti-Stat1 (A–D) antibody and percentages of Stat1- (E) and Stat5-positive cells (F) in the proliferative and prehypertrophic–hypertrophic zones of ACH, TDII and TDI cartilage samples. The increased number of Stat1- and Stat5-positive cells in the prehypertrophic–hypertrophic zone of ACH, TDII and TDI correlates with the severity of the disease. At high magnification, preferential Stat1 staining of the nuclei is visible mostly in TDI (D). Original magnifications: A–D, ×20; inserted squares A–D, ×40. Percentages of Stat1-positive (E) and Stat5-positive (F) cells are expressed as the mean ± SD of three independent counting on 22 different sections; *$P < 0.05$; #Nonsignificant.

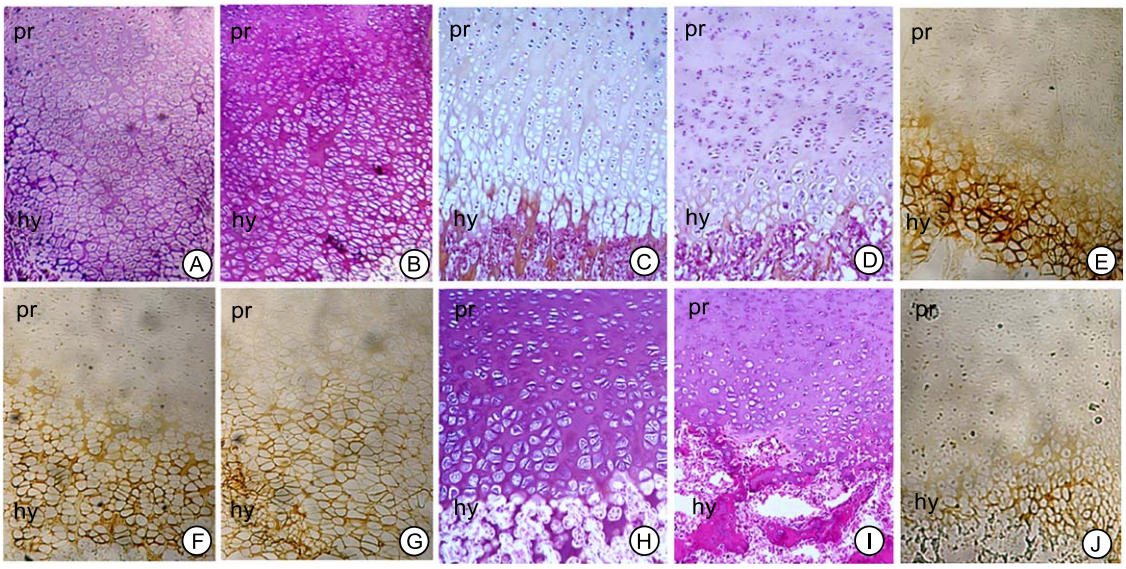
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Fig. 2.

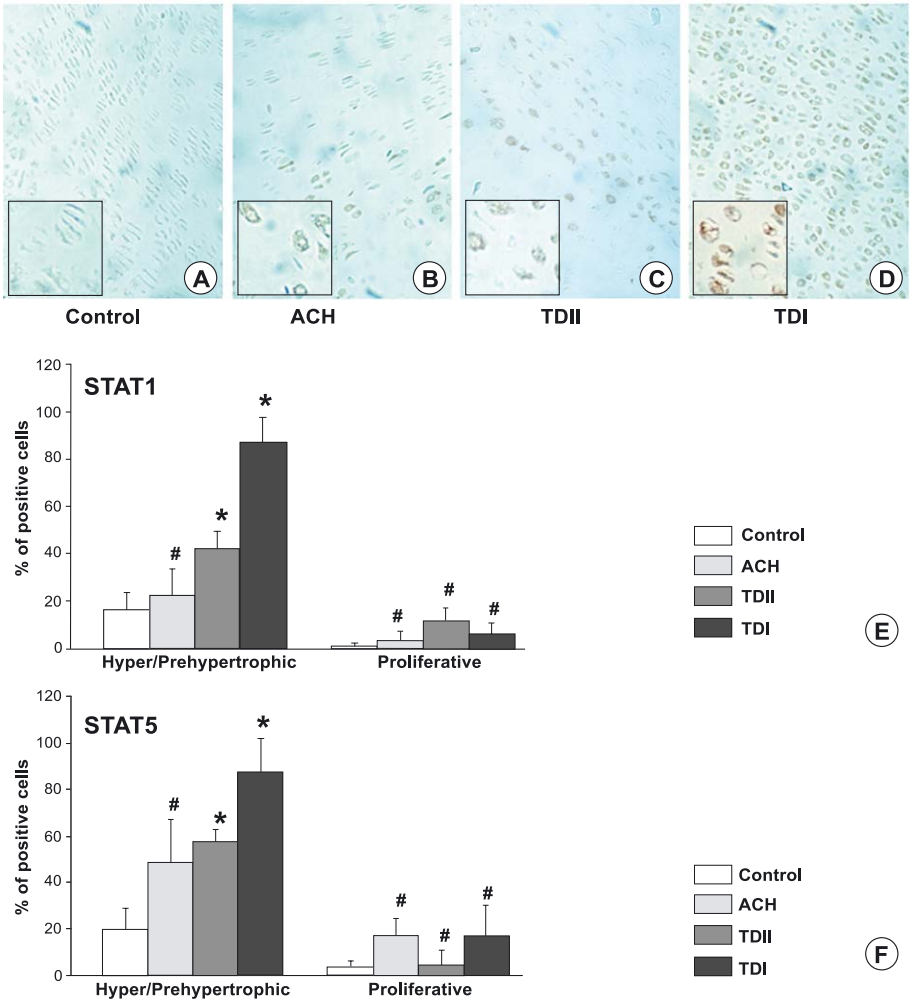
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Fig. 3.

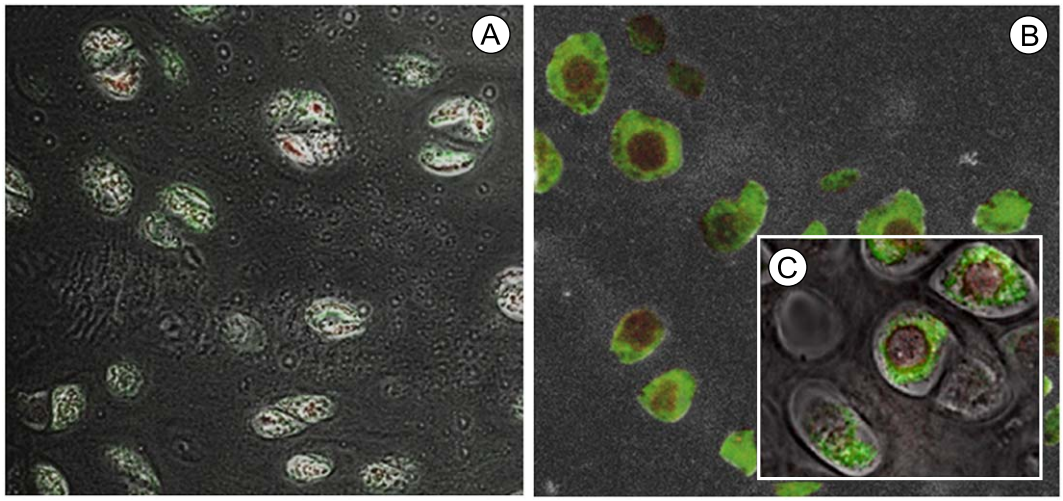

Fig. 4. Double-labeled immunofluorescent staining of control (A) and TDI (B, C) cartilage samples with anti-FGFR3 (FITC) and anti-Stat1 (cy3M) antibodies showing the coexpression of FGFR3 (in green) and Stat1 (in red) in prehypertrophic TDI chondrocytes. Coimmunofluorescence identifies Stat1 only in the nuclei while FGFR3 is present in the cytoplasm. Original magnification: A and B, ×250; C, ×500.

cytoplasmic domain, Sigma Chem.), Stat1, Stat5 (Transduction lab. Lexington, KY), $p21^{Cip1}$ (Santa Cruz, CA) and collagen type X (kindly provided by Dr. W. Horton, Portland, OR) at the following respective concentrations: 1/50, 1/100, 1/100, 1/100 and 1/400. The amplification kit EnVision+ system for visualization of staining was from Dako (Dako

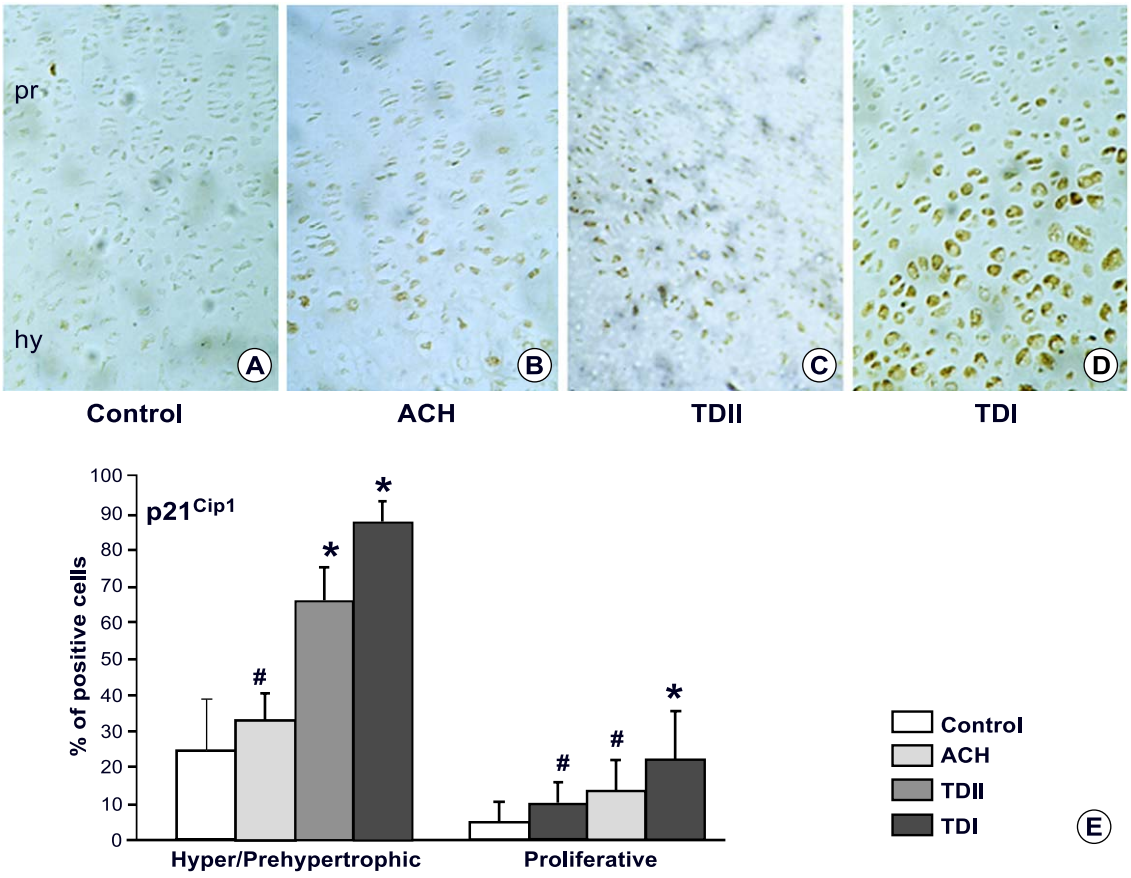

Fig. 5. Immunostaining of growth plates with an anti-$p21^{Cip1}$ antibody (A–D) and percentages of $p21^{Cip1}$-positive cells (E) in the proliferative and prehypertrophic–hypertrophic zones of ACH, TDII and TDI cartilage samples. In TDI, a few $p21^{Cip}$-positive cells are detectable in the lower part of the proliferative zone. Original magnification: A–D, ×20. Percentages of $p21^{Cip1}$-positive cells are expressed as the mean ± SD of three independent counting on 22 different sections; $^{*}P < 0.05$; $^{\#}$Nonsignificant.

Corp. Santa Barbara, CA). No background was detectable when the specific primary antibody was omitted or when the amplification system was used in the absence of antibodies. Stained and unstained cells were counted under the microscope by three independent investigators. At least 200 cells per section were counted and mean values were calculated. Results are expressed as the mean percentage ± SD of antibody positive cells.

For confocal studies, paraformaldehyde-fixed cartilage sections were double-labeled for 2 h at room temperature with anti-FGFR3 and anti-Stat1 antibodies, incubated with FITC-conjugated goat anti-rabbit antibody (FGFR3) or cy3M-conjugated goat anti-mouse antibody (Stat1) for 2 h at room temperature and examined with a Zeiss LSM 510 confocal microscope.

Statistical methods

Statistical comparisons of immunostained cells in cartilage growth plates were made using the Wilcoxon rank-sum test with a probability of less than or equal to 0.05 being considered significant.

Results

Mutation screening in human fetuses

Cartilage samples were obtained from a total of 40 fetuses aged 13–38 weeks of gestation and compared to cartilages from 10 control fetuses aged 12–36 weeks (Table 1). Mutation analyses showed that all fetuses diagnosed as ACH (14/14) carried the recurrent *G380R* mutation. The *K650E* mutation was detected in all TDII fetuses (7/7). The TDI fetuses harbored mutations either in the linker region between IgII and IgIII loops (*R248C*: 13/19 and *S249C*: 3/19) or in the juxtamembrane domain (*Y373C*: 3/19).

Heterozygous FGFR3 mutations cause overexpression and constitutive activation of the endogenous receptor in primary cultured chondrocytes

We had previously shown by in situ hybridization and northern blot analysis that control and TD primary cultured chondrocytes, when seeded at high density, expressed collagen type II and FGFR3 [17], indicating that their differentiated phenotype was maintained. This was further confirmed by alcian blue staining of the matrix (results not shown) and immunoblotting with an anti-collagen type II antibody (Fig. 1).

To assess, at the protein level, the consequences of heterozygous *FGFR3* mutations on the endogenous receptor, cell lysates from control, ACH and TD chondrocytes grown for 5–6 days in primary culture were immunoblotted with anti-FGFR3 and anti-phosphotyrosine antibodies and compared with stably transfected NIH3T3 cells. While stable transfection of wild-type (wt) FGFR3 cDNA in NIH3T3 cells gave rise to two isoforms of 130 and 120 kDa, respectively, corresponding to various degrees of receptor glycosylation, only the fully glycosylated 130-kDa isoform was detected in primary chondrocytes. The intensity of this band was slightly increased in ACH and strongly increased in TDI cells. In the absence of FGF ligand, the receptor was constitutively phosphorylated in TDI cells. No signal was detected in control chondrocytes. FGFR3-transfected NIH3T3 cells in the absence of ligand showed no tyrosine phosphorylation (results not shown). Stimulation with FGF 18 induced phosphorylation of the 130-kDa isoform only (Fig. 1), suggesting that the partially glycosylated isoform (120 kDa) was not present at the cell surface.

Correlation between cartilage growth plate abnormalities and phenotypic severity of the disease

Analysis of cartilage sections from a 13-week-old ACH fetus showed that at this stage of development the ACH growth plate closely resembled that of an age-matched control, although ACH chondrocytes appeared less numerous in some areas (Figs. 2A,B). Immunostaining with an anti-collagen type X antibody (a specific marker of hypertrophic chondrocytes) showed a strong and specific staining of the extracellular matrix in the hypertrophic zone that was undistinguishable from the control (Figs. 2F,G). At later stages (29–35 weeks), HES staining of ACH cartilage growth plates revealed variable degrees of disorganization of the proliferative and hypertrophic zones. Hypertrophic cells appeared smaller than control samples and the columnar arrangement was altered (Figs. 2C,D).

Cartilage defects in TD fetuses were already detectable at mid-stages of development (17–24 weeks) and correlated to phenotypic severity. In TDII fetuses, disorganization of chondrocyte columns was mostly visible in the proliferative zone, hypertrophic cells looked smaller and less numerous than their normal counterparts (Figs. 2C,H). In TDI fetuses, the size of the growth plate was markedly reduced (Figs. 2C,I). Reduction was particularly obvious in the hypertrophic zone with small hypertrophic chondrocytes resembling control prehypertrophic cells and expressing low levels of collagen type X in the matrix (Figs. 2E,J). Columns were totally absent and the line of ossification was distorted. Mutations in either the linker region (*R248C* and *S249C*) or the juxtamembrane domain (*Y373C*) gave similar patterns (results not shown).

Overexpression of Stat1 and Stat5 in the prehypertrophic–hypertrophic zone of ACH and TD growth plates

Constitutively activated FGFR3 in cartilage cells is known to signal through the STAT pathway [14,34]. Expression of STAT proteins in the growth plates of ACH and TD fetuses was thus investigated by immunohistochemical staining with anti-Stat1 (Figs. 3A–E) and

anti-Stat5 antibodies (Fig. 3F). In ACH chondrocytes, the level of expression of both Stat1 and Stat5 proteins was mainly increased in the prehypertrophic–hypertrophic zone. Staining was more striking in TDII samples and reached a maximum in TDI where 80–90% of prehypertrophic–hypertrophic cells was positive and exhibited mostly nuclear staining. In control samples, a faint signal was visible in the proliferative and hypertrophic zones and positive cells showed cytoplasmic staining exclusively. Counting Stat1- and Stat5-positive cells in a series of seven ACH, three TDII, six TDI and six control cartilage sections confirmed microscope examination data (Figs. 3E,F). In the proliferative zone, the number of positive cells was in the range of 2–4%. Higher mean values were found in ACH and TD samples (8–20%), but they were not statistically different from control ($P > 0.05$) due to variations among samples. By contrast, in the prehypertrophic–hypertrophic zones, the number of Stat1- and Stat5-positive cells was statistically increased ($P < 0.01$ and $P < 0.04$, respectively) and directly related to the phenotypic severity (Figs. 3E,F). Further evidence for activation and nuclear translocation of Stat5 in ACH and TD chondrocytes was obtained by immunoblotting primary chondrocyte lysates with antibodies raised against Stat5 and phospho-Stat5. While Stat5 was expressed both in control and patient chondrocytes, phosphorylation of this signaling protein was detectable in ACH and TD cells only (Fig. 1).

Stat1 overexpression in chondrocytes strongly expressing mutant FGFR3 protein

The increased amount of endogenous receptor in primary cultured ACH and TD chondrocytes suggested that cells overexpressing Stat proteins also expressed high levels of the receptor. To test this hypothesis, TDI cartilage sections were double-labeled with anti-FGFR3 and anti-Stat1 antibodies conjugated to fluorescent dyes, then analyzed by confocal microscopy. High amounts of FGFR3 were found in the cytoplasm of TDI prehypertrophic cells. FGFR3-positive TDI chondrocytes also showed high levels of Stat1 in their nuclei (Fig. 4), whereas control chondrocytes from the same zone stained poorly with both antibodies and failed to show Stat1 nuclear staining, further confirming that *FGFR3*-activating mutations lead to nuclear translocation of Stat proteins.

Overexpression of p21Cip1 in ACH and TD chondrocytes

Considering that Stat1 activation induces expression of the cyclin-dependent kinase (cdk) inhibitor $p21^{Cip1}$, we studied expression of the $p21^{Cip1}$ protein at the growth plate level by staining cartilage sections with an anti-$p21^{Cip1}$ antibody (Figs. 5A–D). While only 25% of control prehypertrophic–hypertrophic chondrocytes showed a weak positive staining (Fig. 5E), more than 85% of TDI cells stained positively ($p = 0.01$) and exhibited a much stronger signal than age-matched control chondrocytes (Figs. 5A,D). Less than 5% of control chondrocytes from the proliferative zone showed faint staining (Fig. 5E). In TDI growth plates, the mean number of proliferating cells expressing $p21^{Cip1}$ was significantly increased when compared to control ($p = 0.04$), but the $p21^{Cip1}$-positive cells were found exclusively in the lower part of the proliferative zone adjacent to the prehypertrophic zone. In keeping with this observation, 5–10% of primary cultured TDI chondrocytes stained positively with an anti-$p21^{Cip1}$ antibody whereas immunostaining was almost undetectable in control chondrocytes (results not shown).

Since $p21^{Cip1}$ is an inhibitor of cyclin-dependent kinases (cdk) able to form a quaternary complex with a cyclin, a cdk and the proliferating cell nuclear antigen (PCNA) [39], expression of PCNA and cdk2 in primary cultured chondrocytes was tested. No significant differences among ACH, TDI and control were found (results not shown). Likewise, ACH and TD chondrocytes proliferated normally in the presence of FGF9 or FGF18 (results not shown), further supporting the conclusion that *FGFR3* mutations at mid- and late stages of human prenatal development do not suppress the capacity of chondrocytes to proliferate.

Discussion

The recent engineering of various mouse models aimed at generating skeletal conditions that mimic *FGFR3*-related human chondrodysplasias has brought new insights into the multiple signaling pathways by which FGFR3 may regulate growth and differentiation of long bones [7,8,15,16,18,22,31,40]. However, comparison of mouse models with human fetuses showed that in most animal models, phenotypes at birth were milder than in human (Table 2). Consequently, few studies have focused on the consequences of receptor activation during embryonic development, but most studies were done during the postnatal period. The availability of human samples, therefore, appeared as a valuable tool for analyzing the effects of mutations on cartilage growth plates during prenatal development. At an early developmental stage (13 weeks), the *G380R* mutation causing ACH had no visible effect on cartilage organization, suggesting that constitutive activation of the receptor in ACH chondrocytes was weak and had little influence on the first steps of skeletal development. Consistent with the ultrasound detection of short femurs after 25–27 weeks of gestation, significant histological abnormalities were detected at 29 weeks. This finding was in keeping with the observation that introduction of ACH mutation in the mouse genome resulted in relatively mild phenotypes. Out of four ACH mouse models, only animals overexpressing a human *FGFR3* transgene exhibited a phenotype at birth; skeletal anomalies being mostly visible in homozygous mice

Table 2
Summary of the effects of FGFR3 mutations on the growth plates of mouse models and human fetuses

Phenotype	Mutation	Reference	Method	Prenatal defect	Prenatal period					Postnatal period				
					Hypertrophic zone	Proliferation	STAT1–5 expression	P21 expression	PTHrP–Ihh expression	Hypertrophic zone	Proliferation	STAT1–5 expression	P21–INK4 expression	PTHrP–Ihh expression
Mouse models														
ACH	G380R	16	Transgene	No	n.a.	Unchanged	n.a.	n.a.	n.a.	Reduced	Decreased	Increased	Increased	Decreased
ACH	G375C	18	Gene targeting	No	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	Decreased	n.a.	n.a.	n.a.
ACH	G380R	21	Gene targeting	No	n.a.	n.a.	n.a.	n.a.	n.a.	Reduced	Decreased	n.a.	n.a.	n.a.
ACH	G380R	19	Transgene	Yes	Reduced	n.a.	n.a.	n.a.	n.a.	Reduced	Decreased	n.a.	n.a.	n.a.
SADDAN	K644M	23	Gene targeting	Yes	Reduced	Increased	n.a.	n.a.	n.a.	Reduced	Decreased	n.a.	n.a.	Decreased
TDII	K644E	20	Gene targeting	No	n.a.	n.a.	n.a.	n.a.	n.a.	Reduced	Decreased	Increased	Increased	Decreased
TDII	K644E	17	Gene targeting	Yes	n.a.	Increased	Unchanged	n.a.	Unchanged	Reduced	n.a.	Unchanged	n.a.	Unchanged
TDI	S365C	22	Gene targeting	No	n.a.	n.a.	n.a.	n.a.	n.a.	Reduced	Decreased	Increased	n.a.	Decreased
Human fetuses														
ACH	G380R	This study	–	Yes	Reduced (+)	Unchanged	Increased	Increased	Unchanged					
TDII	K650E	This study	–	Yes	Reduced (2+)	Unchanged	Increased	Increased	Unchanged					
TDI	R248C	This study	–	Yes	Reduced (3+)	Unchanged	Increased	Increased	Unchanged					
TDI	S249C	This study	–	Yes	Reduced (3+)	Unchanged	Increased	Increased	Unchanged					
TDI	Y373C	This study	–	Yes	Reduced (3+)	Unchanged	Increased	Increased	Unchanged					

All results were expressed upon comparison with animals overexpressing the wild-type FGFR3 gene (mouse models) or with control fetuses (human). n.a., not available.

[31]. By contrast, mice targeted for TDII or SADDAN mutations displayed prenatal skeletal defects [15,16] as early as embryonic day 14 in animals harboring the TDII mutation [16]. In human, mutations causing TDI and TDII phenotypes resulted in disorganized cartilage growth plates at 16–17 weeks of development. Since cartilage samples from TD fetuses less than 16 weeks were not available for analysis, we could not determine whether anomalies were present at earlier stages, but it is tempting to speculate that the strong constitutive activation of the receptor caused by TDI mutations [23] might disrupt the first steps of endochondral ossification. Taken together, our observations indicate that at mid- and late stages of fetal development, histological disorganization of the cartilage growth plate is directly correlated to the phenotypic severity of the disease.

Whether *FGFR3* mutations affect chondrocyte proliferation, chondrocyte differentiation or both is still a matter of debate. Indeed, depending on the mouse model, reduced or accelerated chondrocyte proliferation in the prenatal period has been described [8,15,31]. Increased proliferation was observed in TDII and SADDAN models at embryonic day 14 and 15.5, respectively, but proliferation returned to normal at day 18.5. Similarly, primary cultured ACH and TD chondrocytes proliferated normally and responded to the mitogenic effects of FGF9 and FGF18 as efficiently as control chondrocytes [17] (results not shown), suggesting that at late stages of prenatal development, FGFR3 mutations have little effect on the proliferative capacities of chondrocytes both in human and mice, whereas decreased cell proliferation has been reproducibly observed in mouse models after birth (Table 2).

Recent data from a mouse limb culture system [19] and immunohistochemical analysis of TDII and SADDAN mouse models [15,16] strongly suggest that defective differentiation of chondrocytes is the primary cause of dwarfism. Support to this assertion is provided by our observation that terminal differentiation of hypertrophic chondrocytes from ACH and TD fetuses was altered, since they were smaller and less numerous than normal cartilage. Analysis of the STAT signaling pathway in the human growth plate revealed that defective differentiation was also associated with a strong overexpression of Stat1 and Stat5 proteins in prehypertrophic–hypertrophic chondrocytes and a lesser increase in the last arrays of proliferative cells. The extent of overexpression correlated with the phenotypic severity of the disease and high amounts of Stat proteins were detected in TDI chondrocytes coexpressing the activated mutant receptor, further indicating that FGFR3 activation directly accounts for Stat overexpression.

Activation of Stat1 by mutant FGFR3 has been originally documented in vitro [30,34]. Additional experiments with mouse models have shown increased expression of Stat1 and Stat5 in proliferative cells during the postnatal period [7,8,18]. We demonstrate here that Stat overexpression already occurs at prenatal stages. The marked increase of Stat1 and Stat5 expression in the nuclei of human prehypertrophic chondrocytes is evocative of an accelerated cell differentiation. Support to this statement arises from the recent demonstration that FGF signaling in a limb culture system accelerates the onset of hypertrophic differentiation [19] and from the finding, using cDNA microarrays, that FGF stimulation of RCS cells increases Stat5a expression and promotes hypertrophic differentiation [12]. Additionally, the weak expression of Stats in the proliferative zone argues against a direct involvement of Stat1 in chondrocyte proliferation as previously suggested [30].

Stat1 is known to induce expression of $p21^{Cip1}$, a member of the Cip–Kip family of Cdk inhibitors regulating cell cycle by blocking the G1-S transition [10,32]. Immunostaining of human cartilage growth plates with an antibody to $p21^{Cip1}$ disclosed strong overexpression of this protein mostly in prehypertrophic–hypertrophic cells of ACH and TD fetuses and to a lesser extent in the last layers of TD I proliferative cells adjacent to the prehypertrophic zone. In normal mouse cartilage, $p21^{Cip1}$ is expressed in postmitotic hypertrophic chondrocytes but not in proliferative cells [26]. Recent results have further demonstrated its role in chondrocyte differentiation both in vivo [33] and in vitro [2,22]. Notably, transient increase of $p21^{Cip1}$ expression was observed in differentiating ATDC5 chondrogenic cells but not in undifferentiated cells, indicating that $p21^{Cip1}$ is required for progression of chondrogenic differentiation [24]. We speculate that $p21^{Cip1}$ overexpression at the junction between the proliferative and the prehypertrophic zones in ACH and TD fetuses could reflect premature exit of proliferative cells from the cell cycle, leading to accelerated chondrocyte differentiation. Our results are at variance with an in vitro study showing that stimulation of embryonic mouse limb explants with FGF2 induced generalized expression of $p21^{Cip1}$ in the whole growth plate [1]. The discrepancy between the two studies might be related to the use of FGF2 that binds not only to FGFR3 but also to FGFR1 which is expressed in mesenchymal and osteoblastic cells [6,25].

In summary, the availability of cartilage samples from human fetuses has enabled us to investigate the consequences of *FGFR3* mutations on endochondral ossification during the mid- and late stages of fetal development which remain poorly documented in mouse models. Human and mouse data suggest that significant differences exist between the prenatal and the postnatal stages in term of proliferation and differentiation of cartilage cells. In the most severe phenotypes (TD), prenatal abnormalities may be present at the first steps of endochondral ossification (E12–E14 in mice and 7–8 weeks in human) and would primarily affect chondrocyte maturation or differentiation. In the postnatal period, defective differentiation seems more striking and is accompanied by defective chondrocyte proliferation. Abnormal chondrocyte differentiation appears to be the main cause of bone growth retardation in *FGFR3*-related chondrodysplasias.

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