Human immortalized chondrocytes carrying heterozygous FGFR3 mutations: An in vitro model to study chondrodysplasias

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Abstract **Achondroplasia and thanatophoric dysplasia are human chondrodysplasias caused by mutations in the fibroblast growth factor receptor 3 (FGFR3) gene. We have developed an immortalized human chondrocyte culture model to study the regulation of chondrocyte functions. One control and eight mutant chondrocytic lines expressing different FGFR3 heterozygous mutations were obtained. FGFR3 signaling pathways were modified in the mutant lines as revealed by the constitutive activation of the STAT pathway and an increased level of P21[WAF1/CIP1] protein. This model will be useful for the study of FGFR3 function in cartilage studies and future therapeutic approaches in chondrodysplasias.**
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1. Introduction

Fibroblast growth factor receptor 3 (FGFR3) is a tyrosine kinase receptor that mediates the action of various Fibroblast Growth Factors (FGF). Activating FGFR3 mutations in humans cause a broad clinical spectrum of chondrodysplasias ranging from hypochondrondoplasia (HCH) and achondroplasia (ACH), to lethal thanatophoric dysplasia (TD) [1–6]. Mutations in FGFR3 can result in the constitutive activation of the receptor or the stabilization of the dimerized monomers [7,8]. We have previously shown that the proliferating and hypertrophic chondrocyte zones of the human TD growth plate are markedly reduced and disorganized, suggesting that both chondrocyte proliferation and differentiation are altered by FGFR3 mutations [9,10]. Chondrocyte proliferation and differentiation are known to require activation of various signaling proteins, including STATs, MAPK ERK1/2, phospholipase Cγ, protein kinase C and AKT [11–14]. Mutations in FGFR3 have been shown to trigger the constitutive activation of the STAT(s) signaling pathway and increased expression of the cell-cycle inhibitor p21[WAF1/CIP1] in mouse and human cartilage models [10,15]. The MAP kinase pathway is also constitutively activated by FGFR3 mutations [15].

Chondrocytes derived from human growth plate are difficult to cultivate as they rapidly loose their round shape and de-differentiate into a fibroblast-like cell type when grown on plastic thus forming a monolayer of flattened cells.

In order to overcome the de-differentiation of primary chondrocytes we have developed a human chondrocyte culture model for human chondrodysplasia studies. Primary chondrocytes from normal and chondrodysplasia fetuses were immortalized by transfection using plasmid DNA, expressing origin defective simian virus 40 (SV40) containing large T antigen.

After several rounds of passaging in culture, the immortalized chondrocytes still retain chondrocytic morphology and proliferate in monolayer culture. These human chondrocytic lines carry and express wild-type and mutant FGFR3 and can consequently serve to analyze FGFR3 signaling pathways in human chondrodysplasias.

2. Materials and methods

2.1. Cartilage samples and cell culture

Human chondrocytes were isolated from fetal growth plate cartilage derived from medically aborted control (1 fetus), ACH (1 fetus) and TD (7 fetuses) following the informed consent of the parents. Pregnancies were legally terminated after ultrasonographic and X-ray detection of chondrodysplasia. Chondrocytes were isolated as described previously [9], plated at $2 \times 10^6/10 \, cm$ dish, and cultured during four days prior to transfection in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen) in 5% CO_2 at 37 °C.

Immortalized human chondrocytes were obtained by transfection with Simian virus 40 largeT-antigen (SV40-TAg) using Fugene 6 (Roche) and the neomycin-resistance selection marker (neo[R]). The cells were selected using fresh medium containing G418 (500 μg/ml geneticin; Invitrogen). Selection in G418 was maintained for 4 weeks, and after an initial 80% reduction in cell density, G418-resistant cells repopulated the dishes. The transfected cells were subcloned and selected according to chondrocytic morphology.

2.2. DNA sequencing and mutation analyses

The screening of FGFR3 mutations in immortalized human chondrocytes was performed by direct sequencing of amplification products as described previously [16]. All the FGFR3 coding regions from control and mutant strains were sequenced to confirm the absence of additional polymorphisms or mutations.

2.3. Reverse transcriptase PCR

Extraction of primary and immortalized chondrocyte RNA was performed using the RNeasy extraction kit (Qiagen). Total RNA (0.2 μg) was reverse transcribed using an RNA PCR Core kit (Applied-Biosystems). Following an initial denaturation at 95 °C for 2 min., the cDNA was amplified in the Gen AmpPCR system 9600 (Perkin–Elmer) using

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Table 1
FGFR3 mutations identified in human immortalized and primary chondrocytes

No.	Cell line	Mutation	Age of pregnancy (weeks)	Phenotype	FGFR3 domain
Immortalized chondrocytes					
1	GU-15	–	26	Control	–
2	BL-2.2	G380R	32	ACH	TM domain
3	TU-4.3	R248C	19	TDI	EC domain
4	BA-2.7	S249C	21	TDI	EC domain
5	DR-2.1.10	G370C	24	TDI	EC domain
6	BD-1.11	Y373C	26	TDI	EC domain
7	MA-1.8.4	Y373C	15	TDI	EC domain
8	GE-1.2	K650E	14	TDII	TKII domain
9	BR-1.13	X807S	19	TDI	Stop position
Primary chondrocytes					
10	GE	K650E	14	TDII	TKII domain
11	BE	R248C	18	TDI	EC domain

35 cycles of 15 s at 95 °C, 30 s at 60 °C, 7 min at 72 °C, for GAPDH, Biglycan, Decorin, Osteocalcin, Osteopontin, type II Collagen, Aggrecan, type I Collagen, Matrix Metalloprotein 3 (MMP-3) [17] and SOX9 primers [18]. The mRNAs isolated from primary human chondrocytes served as a positive control.

2.4. Immunoprecipitation and immunoblotting

Immortalized chondrocytes were lysed in RIPA buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% Nonidet P-40, supplemented with protease and phosphatase inhibitors) and clarified by centrifugation at $12\,000 \times g$ for 20 min. The lysates were subjected to immunoprecipitation for 20 h at 4 °C with anti-FGFR3 C-terminus (Sigma), anti-STAT3^{Ptyr705}/STAT3 (Cell Signaling), anti-FGFR3 (Sigma) antibodies using protein G agarose (Roche) and analyzed by SDS–PAGE (NUPAGE 4–12% Bis–Tris Gel-Invitrogen) and Western blots. Protein bands were visualized using secondary antibodies coupled to horseradish peroxidase and detected by chemiluminescence (ECL, Amersham Pharmacia Biotech) according to the manufacturer's instructions. PVDF membranes were stripped in 2% SDS, 100 mM β-mercaptoethanol, 50 mM Tris, pH 6.8. Lysates were immunoblotted with P21$^{WAP/CIP1}$ (Transduction Laboratories), ERK1/2-P (Cell Signaling) and ERK1/2 antibodies (Sigma). Cells were treated with Human FGF18 (100 ng/ml; Peprotech) and heparin (10 μg/ml) for the indicated times. All experiments were performed at least three times.

2.5. Immunofluorescence

Immortalized chondrocytes were fixed in 4% PFA for 20 min, permeabilized in 0.1% triton X100/1XPBS and blocked in 10% normal sheep serum. Incubation with primary antibodies: anti-FGFR3 at 1/100 (Sigma) was for 1 h at room temperature followed by incubation with secondary antibodies Alexa488 at 1/400 (Molecular Probes). Cells were covered with mounting solution (Vector) and examined using an Olympus IX2-UCB microscope.

3. Results

3.1. Molecular genotyping of the immortalized chondrocytes

Nine lines of immortalized Human chondrocytes were obtained by sub-cloning: one control and eight mutant chondrocytic lines. Sequencing of the coding regions of the FGFR3 gene in the eight sub-cloned mutant chondrocytic lines detected various heterozygous mutations in several domains of the FGFR3 protein. One cell line carried the achondroplasia mutation (G380R) located in the transmembrane domain, five cell lines carried thanatophoric dysplasia type I (TDI) mutations (R248C, S249C, G370C, Y373C) located in the extracel-
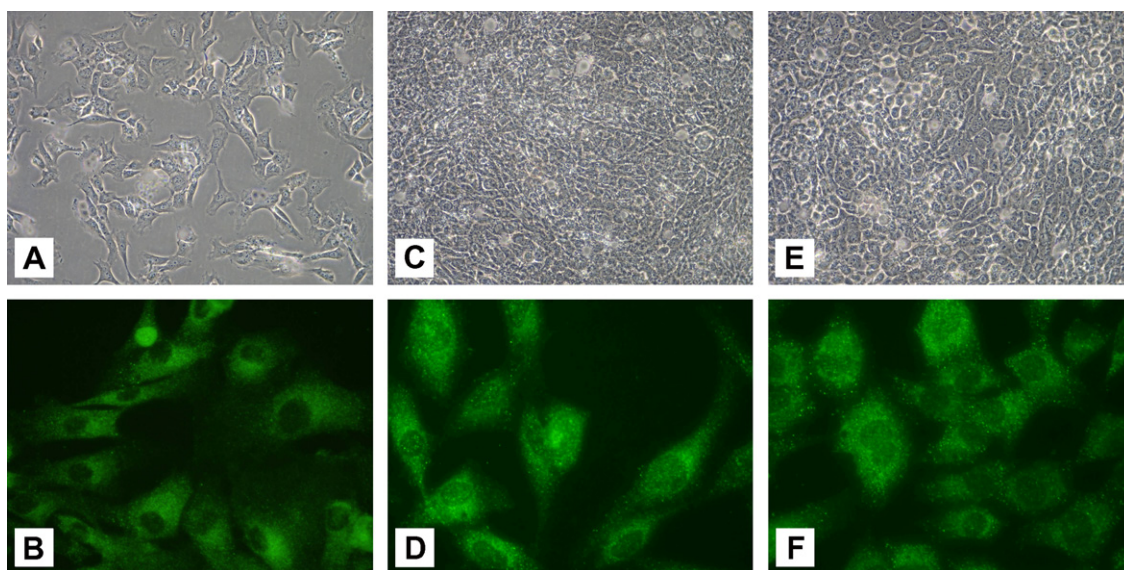

Fig. 1. Morphology of primary and immortalized chondrocytes and FGFR3 immunostaining. (A, B) Primary chondrocyte (BE). (C, D) Control immortalized chondrocytes (GU-15). (E, F) Mutant immortalized chondrocytes (GE-1.2). (A, C, E) Phase contrast microscopy. (B, D, F) Immunofluorescence staining of primary (BE), immortalized (GU-15) and (GE-1.2) chondrocytes.

lular domain. One cell line carried the thanatophoric dysplasia type II (TDII) mutation (K650E) located in the tyrosine kinase II domain. Finally one cell line carried the TDI (X807S) mutation that abolishes the terminal stop codon of FGFR3 (Table 1).

3.2. Molecular phenotyping of the immortalized chondrocytes

The nine immortalized chondrocyte lines were morphologically similar to the primary chondrocytes. When grown in monolayer, they conserved a homogenous polygonal shape and did not have a flattened fibroblast-like shape in keeping with de-differentiation (Fig. 1A). Observed by phase contrast microscopy the control and mutant cell lines exhibited the same phenotype in culture (Fig. 1C, E). The localization of FGFR3 proteins in primary and immortalized chondrocytes was investigated by using an antibody raised against the C-terminal end of the protein. The FGFR3 protein was detected in the cytoplasm of both mutant primary chondrocytes and mutant cell lines (Fig. 1B, D, F).

In order to confirm the chondrogenic differentiation of the cell lines, we analyzed the expression of chondrocyte-specific genes, including SOX9, a key transcription factor of the chondrocytic phenotype [19,20]. This gene was expressed at a high level in control and mutant cell lines and in the corresponding primary chondrocytes, as determined by RT-PCR (Fig. 2A). The expression of the extracellular matrix protein gene COL2A1 in primary chondrocytes was slightly higher than that of COL2A1 mRNA in several cell lines (Fig. 2B). COL1A1 mRNA was found in some cell lines. Moreover, the majority of non-collagenous matrix genes, including, biglycan, aggrecan and decorin were detected in all cell lines and in the corresponding primary chondrocytes (Fig. 2B). In lines with RT-PCR results, the presence of proteoglycan in the extracellular matrix was confirmed by alcian blue staining (data not shown). Genes of matrix turnover such as Matrix Metalloproteinase 3 (MMP3), the matrix-degrading protease, and osteocalcin were also expressed both in cell lines and in primary chondrocytes (Fig. 2B). The chondrocytic lines were kept in culture up to passage 20. During this period, the cell lines displayed a morphologic and phenotypic stability.

3.3. High FGFR3 expression in the mutant cell lines and $P21^{WAF1/CIP1}$ induction

None of the missense FGFR3 mutations studied altered protein synthesis. Immunoprecipitation using an anti-FGFR3 antibody, detected the fully glycosylated FGFR3 protein (130 kDa) in both control and mutant lines and we did not observe any degradation of the mutant FGFR3 compared to wild-type FGFR3 proteins (Fig. 3A). For the cell line carrying the stop codon mutation (X807S) and encoding an elongated C-terminal domain, the antibody directed against the FGFR3 C-Terminal end failed to detect the protein with the predicted 141 amino acid extension (Fig. 3A). However, we cannot exclude a degradation of the elongated protein.

We have previously reported an increased expression of $P21^{WAF1/CIP1}$ in human TD and ACH growth plates [10]. This feature has been also reported in PC12 cell lines carrying TDII mutations [11]. Here we give support to this observation by immunoblot analyses using an anti $P21^{WAF1/CIP1}$ antibody and show this up-regulation in all mutant cell lines (Fig. 3B). $P21^{WAF1/CIP1}$ an inhibitor of cyclin-dependent kinases, is
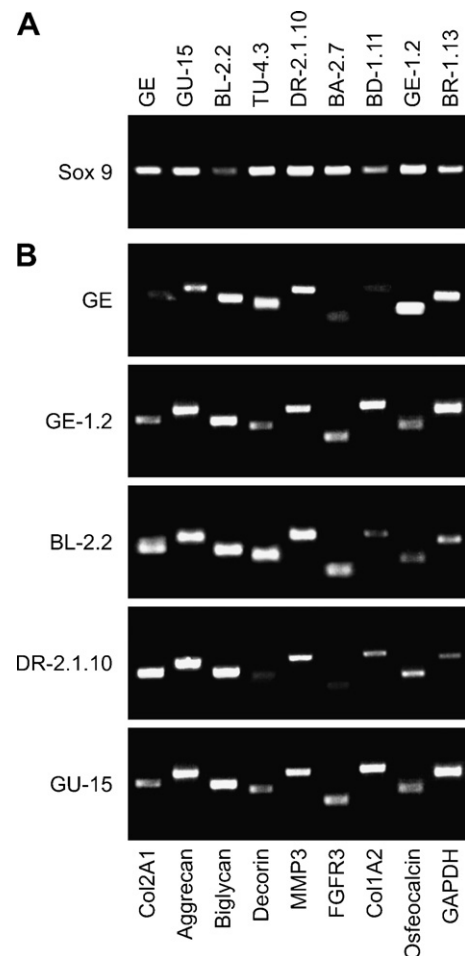

Fig. 2. mRNA expression of matrix protein. (A) Analysis of SOX9 mRNA by reverse transcriptase polymerase chain reaction (RT-PCR) in primary (GE) and immortalized lines (GU-15, BL-2.2, TU-4.3, DR-2.1.10, BA-2.7, BD-1.11, GE-1.2, BR-1.13). (B) Analysis of mRNAs by (RT-PCR) in primary (GE) and immortalized lines (GE-1.2, BL 2.2, DR 2.1.10, GU 1.5).
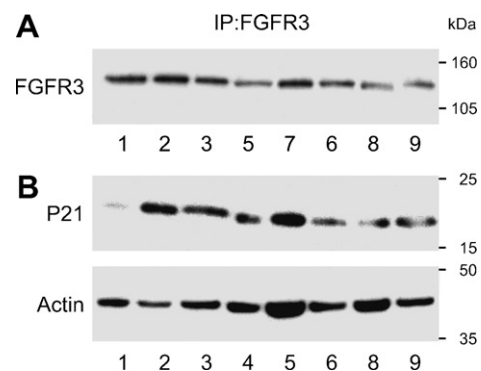

Fig. 3. FGFR3 and $P21^{WAF1/CIP1}$ expression in immortalized chondrocytes. (A) Immunoprecipitation by anti-FGFR3 antibody (C-terminal). Western blot analyses of protein lysates from immortalized chondrocytes: [GU-15 (1), BL-2.2 (2), TU-4.3 (3), DR-2.1.10 (5), MA-1.8.4 (7), BD-1.11 (6), GE-1.2 (8) BR-1.13 (9)] with an anti-FGFR3 antibody. (B) Western blot analyses of protein lysates from immortalized chondrocytes: [GU-15 (1), BL-2.2 (2), TU-4.3 (3), BA-2.7 (4), DR-2.1.10 (5), BD-1.11 (6), GE-1.2 (8), BR-1.13 (9)] with an $P21^{WAF1/CIP1}$ antibody. Actin was used as a loading control.

known to trigger cell growth arrest, is a downstream target of STAT and MAP kinase signaling and is involved in FGFR3 chondrodysplasias.

3.4. Activation of the STAT pathway in mutant chondrocyte lines

Constitutive activation of the STAT pathway by mutant FGFR3 has been documented previously, in a mouse model and in human pathological cartilage [15,21]. We have reported STAT1, STAT3, STAT5 activation in FGFR3 mutations in correlation with disease severity [10]. We have also observed a constitutive activation of the STAT pathway in the mutant cell lines. Indeed, similarly to the mutant primary chondrocytes, the mutant cell lines exhibited a high level of ligand-independant STAT3 phosphorylation. Moreover an overexpression of STAT3 protein was observed in all mutant lines (Fig. 4A). This increase was directly related to the phenotypic severity as previously observed in ACH or TDI growth plates [10].

3.5. FGF18 activates the MAP kinase pathway

The activation of the MAP kinase pathway was tested by studying the level of ERK1/2 phosphorylation following stimulation by FGF18. In the absence of ligand, no phosphorylated ERK1/2 was detected in control or mutant cell lines in
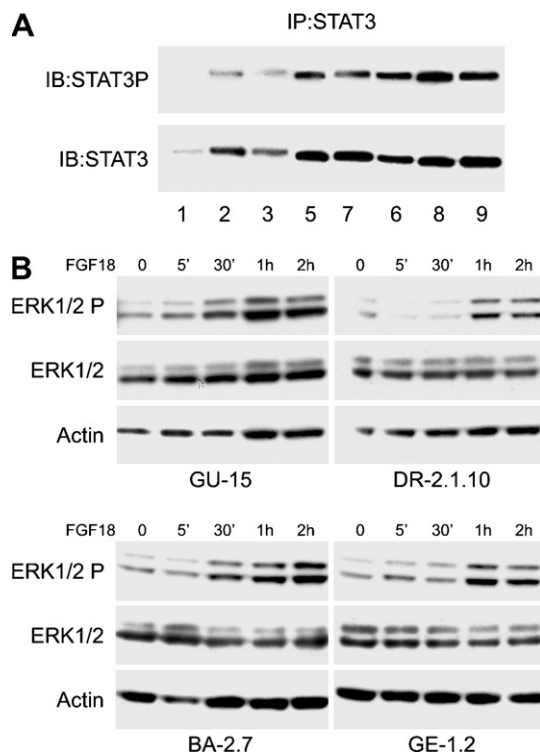

Fig. 4. STAT3 activation in immortalized chondrocytes. (A) STAT3 phosphorylation was assessed by immunoprecipation by anti-STAT3. Membranes were blotted with STAT3-P, stripped and reprobed with STAT-3 antibody GU-15 (1), BL-2.2 (2), TU-4.3 (3), DR-2.1.10 (5), MA-1.8.4 (7), BD-1.11 (6), GE-1.2 (8) BR-1.13 (9). (B) Immunodetection of activated ERK1/2 in immortalized chondrocytes. Duration of ERK1/2 activation in GU-15, DR-2.1.10, BA-2.7, GE-1.2 lines by FGF18 (100 ng/ml). Detection of P-ERK1/2 and ERK1/2 with anti P-ERK1/2 and ERK1/2 antibodies. Actin was used as a loading control. All the cells were depleted during 24 h before FGF18 stimulation.

Fig. 4B. The immortalized chondrocytes (lines 1–8) produced high levels of phosphorylated ERK1/2 after thirty minutes or one hour and two hours of FGF18 stimulation (Fig. 4B), except for line 9 carrying the X807S mutation (data not shown).

4. Discussion

Despite recent advances in understanding the role of FGFR3 in skeletal development, the signaling pathways that mediate its actions remain incompletely understood. In order to investigate the impact of the FGFR3 mutations on the signaling pathways, several cellular models including PC12, HEK293, ATDC5 and RCS [11,22–25] have been used but to date, no human chondrocyte cell line expressing a mutant FGFR3 receptor has been described. The cell lines reported here are the first human immortalized chondrocytes to be characterized. Several immortalized chondrocyte lines derived from various species have been shown to retain the ability to synthesize sulfated proteoglycans [26–29] but the expression of type II collagen appears to be the most reliable criterium of chondrocyte differentiation [29]. In order to determine whether our human chondrocyte cell lines could be regarded as a relevant and reproducible model of chondrocyte differentiation, we tested the expression of proteoglycan and collagens in our cells. Aggrecan (a large aggregating proteoglycan), biglycan and decorin (small proteoglycans) are essential components of matrix cartilage and were expressed in large amounts in the immortalized cell lines. Our control and mutant human chondrocyte lines also retained the ability to synthesize type II collagen, and SOX9. A transcription factor that regulates chondrogenesis, SOX9, is expressed in the normal growth plate, articular cartilage and the immortalized cell lines [30]. The immortalized lines conserved a chondrocytic phenotype and expressed type II collagen, aggrecan, and SOX9. All these observations support the view that our chondrocytic lines closely resemble primary chondrocytes.

Our mutant chondrocyte lines co-produced the mutant and the wild-type FGFR3 proteins (Table 1). Yet the morphology of the immortalized chondrocyte lines was not modified as a result of the various heterozygous FGFR3 mutations. Further immunostaining and confocal microscopy experiments are needed to visualize the localization of the mutant receptor in the immortalized chondrocytes. In accordance with previous studies, the preliminary experiments reported here, suggest that FGF signaling in mutant chondrocyte lines can increase the expression of STAT and induce the expression of the cell-cycle inhibitor P21$^{WAF1/CIP1}$. There is strong evidence that STAT and P21$^{WAF1/CIP1}$ activation is critical to chondrodysplasia. The contribution of the MAP kinase pathway to the pathogenesis remains an open question: although in mice, the MAP kinase pathway is activated in a constitutive manner in the presence of FGFR3 mutations [14] our data show an absence of constitutive activation in human immortalized chondrocytic lines and primary chondrocytes [9]. The absence of MAP kinase activation, in the mutant line encoding an elongated C-terminal domain, is intriguing and justifies being the subject of further study. It is probable that the wild-type and the mutant proteins play a distinct role in the regulation of chondrocyte proliferation and differentiation and consequently, activate various signaling pathways differently.

So far, it has been difficult to assess the immediate effects of FGFR3 mutations and to evaluate the functional significance of MAP kinase and STAT signaling upon hypertrophic differentiation through biochemical manipulations in cells. The human immortalized chondrocytic cell lines described here will shed light on the signaling pathways downstream to FGFR3.

In conclusion, the human chondrocyte lines reported here seem relevant to the study of pathophysiological and therapeutic approaches of chondrodysplasias.

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