

Sustained ERK1/2 but not STAT1 or 3 activation is required for thanatophoric dysplasia phenotypes in PC12 cells

Nakisa Nowroozi^{1,†}, Simona Raffioni^{1,†}, Tracy Wang¹, Barbara L. Apostol¹, Ralph A. Bradshaw² and Leslie Michels Thompson^{1,3,*}

¹Department of Psychiatry and Human Behavior, 2121 Gillespie, ²Department of Physiology and Biophysics, 346-D Medical Sciences I and ³Department of Biological Chemistry, D240 Medical Sciences I, University of California at Irvine, Irvine, CA 92697, USA

Received February 1, 2005; Revised April 5, 2005; Accepted April 11, 2005

Mutations in fibroblast growth factor receptor 3 (FGFR3) cause the most common genetic form of short-limbed dwarfism, achondroplasia (ACH), as well as neonatal lethal forms, thanatophoric dysplasia (TD) I and II. The causative mutations induce graded levels of constitutive activation of the receptor that correspond to the severity of the disorder, resulting in premature entry into hypertrophic differentiation and reduced proliferation of chondrocytes in developing cartilage. Although FGFR3 promotes growth in most tissues, it is a negative regulator of endochondral bone growth. Several signaling pathways have been implicated in these skeletal disorders including the Ras/MEK/ERK pathway and the JAK/STAT, the latter in the most severe phenotypes, however their functional relevance remains incompletely understood. Using PC12 cell lines stably expressing inducible mutant receptors containing the TDII mutation, K650E, sustained activation of ERK1/2 and activation of STAT1 and STAT3, but not STAT5, is observed in the absence of ligand. This activation leads to neurite outgrowth, a phenotypic readout of constitutive receptor activity, and sustained ERK1/2 activity is required for this ligand-independent differentiation. To assess the functional relevance of STAT activation induced by the mutant receptor, STATs were specifically downregulated using RNA-interference. Silencing of STAT1 or 3 independently or in combination had no significant effect on ligand-independent neurite outgrowth, ERK1/2 activation or p21^{WAF1/CIP1} protein levels. These results support a model in which sustained activation of ERK1/2 is a key regulator of the increased transition to hypertrophic differentiation of the growth plate, whereas activation of STATs 1 and 3 is not required.

INTRODUCTION

Activating mutations in the fibroblast growth factor receptor (FGFR) 3 gene cause several skeletal dysplasias, including achondroplasia (ACH), the most common form of human short-limb dwarfism (1,2), hypochondroplasia, a milder form of dwarfism (3) and thanatophoric dysplasia (TD) types I and II, which are neonatal lethal forms of dwarfism (4,5). The FGFR family members (FGFR1–4) are receptor tyrosine kinases that function in a broad spectrum of developmental and regenerative processes and are key regulators of skeletal growth and differentiation. Fibroblast growth factors (FGFs),

which now number over 20, act as ligands for FGFR signaling (reviewed in 6) and generally also require heparin or a heparin-like substance to form a stable ligand/receptor complex (7,8). The complexity of the system is further amplified through alternative RNA splicing of the receptors leading to altered ligand specificity.

Endochondral ossification, in which bone replaces pre-existing cartilage, is the predominant mechanism of longitudinal bone growth (9). During this process, bones grow longer at the epiphyseal growth plates, where chondrocytes progress through a series of differentiation stages. Various signaling molecules including FGFs have been shown to regulate and

*To whom correspondence should be addressed at: Department of Psychiatry and Human Behavior, 2121 Gillespie, University of California at Irvine, Irvine, CA 92697-4260, USA. Tel: +1 9498246756; Fax: +1 9498242577; Email: lmthomps@uci.edu

[†]The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

coordinate this complex process (10–13). Expression of the activated FGFR3 mutants in mice reproduces features of the dwarf phenotype of these skeletal diseases (12,14–19), whereas lack of FGFR3 in mice causes skeletal overgrowth, indicating that FGFR3 signaling negatively regulates endochondral bone growth (20,21).

Despite recent advances in understanding the roles of FGFs and FGFRs in skeletal development, the intracellular signals that mediate these actions are still not fully defined. FGFs have been shown to activate multiple signaling proteins, including STATs, the MAPKs p38 and ERK1/2, phospholipase C γ , protein kinase C, Src, phosphatidylinositol 3-kinase (PI3K) and AKT (5,22–30). Both ACH and TD mutations cause graded constitutive activation of the receptor, as evidenced by ligand-independent receptor tyrosine phosphorylation and downstream cellular effects, the extent of which correlate with the severity of the phenotype (31–35). Among these, STAT1 activation by mutant FGFR3 is proposed to be involved in the TD phenotypes based on its nuclear localization, indicative of activation, phosphorylation. Increased expression of the cell-cycle inhibitor p21^{WAF1/CIP1} (22,24,36,37), which can be induced by STAT1 activation, in cartilage of mouse model and human TD tissue (14,22,37) observed. Further, the TD mutation appears to hamper complete maturation of FGFR3, leading to activation of STAT1 from the endoplasmic reticulum (33,38,39).

In addition to STAT activation, the MEK/ERK (ERK1/2) signaling pathway is activated in a constitutive manner in the presence of FGFR3 mutations that cause TD (30,33,37). Recent studies suggest that although FGF stimulation of normal FGFR3 activates a spectrum of signal transduction molecules traditionally involved in FGFR signaling, the prolonged activation of ERK may be a critical event for growth arrest and premature differentiation in chondrocytes (23,40,41). ERK1/2 activation is important for both cell proliferation and differentiation, and the strength or duration of this signal is suggested to determine the cellular outcome.

Here, we investigate the functional significance of STAT1 and 3 on the activation of signaling and neurite outgrowth in PC12 cells that, like dividing chondrocytes, must enter a growth arrest/differentiation pathway. Using the FGFR3^{K650E} mutation that causes a neonatal lethal form of dwarfism, TD type II, as a model to understand the milder and potentially treatable ACH. Chemical inhibition of ERK1/2 and acute knockdown of STATs confirm the key role of sustained ERK1/2 activation in the manifestation of ligand-independent phenotypes while demonstrating that STAT activation is not required for premature entry into the differentiation pathway.

RESULTS

Gene expression profiling of PFR3^{K650E} expressing cells suggests constitutive activation of signaling pathways and shows p21 induction

Through multiple lines of research, knowledge regarding the signaling networks and signaling molecules activated by ligand stimulation of FGFRs has emerged (6,42). However, the question remains as to whether constitutive activation of FGFR3, such as that observed with TD activating mutations,

stimulates the activity of novel pathways or whether known pathways are simply activated in the absence of ligand. That is, are qualitative or quantitative differences are observed? To investigate signaling pathways activated by FGFR3 mutations through an analysis of altered expression of downstream transcriptional targets, we performed gene expression profiling of stably transfected inducible PC12 cells expressing a chimeric receptor (PFR3) consisting of the extracellular domain of human platelet derived growth factor receptor (PDGFR) and the transmembrane and intracellular domains of human FGFR3 (33). To our knowledge, no other inducible cell lines expressing the mutant receptor have been described. Cells expressing wild-type receptor under the control of a zinc inducible metallothionein (MT) promoter (PFR3), produce neurites only after the addition of the ligand platelet derived growth factor (PDGF) in the presence of Zn²⁺, which induces expression of the stably transfected gene. In contrast, cells expressing a chimeric receptor containing the K650E mutation under the control of a MT promoter (PFR3^{K650E}) grow neurites in the presence of inducer (i.e. Zn²⁺) alone, indicating ligand-independent activation of this receptor (33). Exogenous addition of PDGF further enhanced the length of neurites in these cells, suggesting that the mutant receptors remain ligand-responsive.

Rat Affymetrix GeneChips were performed on induced PFR3^{K650E} versus induced PFR3 cells and comparison analyses were generated (see Supplementary Material). One of the upregulated genes, p21^{WAF1/CIP1}, induced 1.7-fold, was confirmed by both northern (1.4-fold) and western blot analyses (Fig. 1). This gene was of particular interest as p21^{WAF1/CIP1} induction triggers cell growth arrest and is a downstream target of STAT1 and ERK1/2 signaling, implicated in FGFR3-mediated skeletal dysplasias. Indeed, expression changes of the majority of the altered genes are consistent with activation of ERK and/or STAT signaling, including genes such as *egr1*, p21^{WAF1/CIP1} and MAP kinase phosphatase 1 (*MKP-1*). Several induced genes are also in common with those identified by gene expression profiling in the presence of FGF in rat chondrosarcoma (RCS) cells, a chondrocyte cell line that primarily expresses the FGFR3 form of the FGF receptor types (43). Therefore, gene expression profiling of ligand-independent signaling is most consistent with an enhancement of normal FGFR3 signaling that can occur in the presence of ligand, as opposed to the activation of novel signaling pathways.

ERK1/2 phosphorylation is sustained in PFR3^{K650E} cell lines

Sustained ERK activation, as opposed to transient activation, is an inherent aspect of differentiation (neurite outgrowth) in PC12 cells (reviewed in 44). Gene expression profiling and published studies suggest that ERK and STAT signaling may contribute to altered cell cycle regulation and ligand-independent neurite outgrowth mediated by the K650E mutation. We have previously shown that upon ligand binding, both PFR3 and PFR3^{K650E} activate ERK1/2 and that PFR3^{K650E} can do so in a ligand-independent manner (33). Here, we find that the mutant form (K650E) of the receptor produces a highly sustained ERK1/2 activation even after

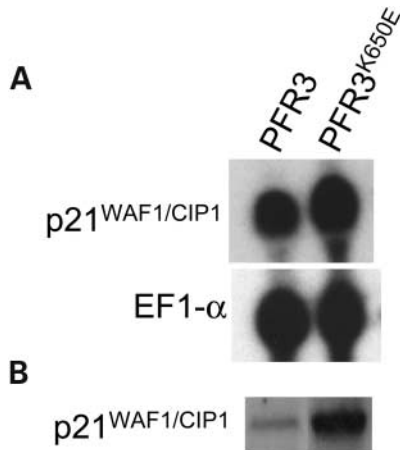


Figure 1. p21^{WAF1/CIP1} shows increased expression in PFR3^{K650E} relative to PFR3 cells. (A) Northern blot analysis of RNA from mutant and wild-type cells induced for 24 h and probed with p21^{WAF1/CIP1} cDNA. Membrane was stripped and re-probed with EF1 α as a loading control. (B) Western blot analyses of protein lysates from cells (A) probed with an anti-p21^{WAF1/CIP1} antibody. Equal loading was confirmed by stripping the blot and re-probing with anti-actin antibody (data not shown). For a description see Materials and Methods.

1 h of PDGF treatment, whereas in wild-type expressing cells, ERK1/2 activity decreases by 1 h post-PDGF addition (Fig. 2A). Using anti-phospho-ERK1/2 western blots of PFR3^{K650E} cell lysates, phosphorylation and activation of ERK1 and ERK2 is present in the mutant cell at low levels even before the addition of inducer, suggesting some leakiness within the system. However, neurite outgrowth, a phenotypic readout for productive receptor activity, is only observed upon synthesis of new receptor following induction. Therefore mutant FGFR3 activates ERK1/2 in a ligand-independent manner and maintains this activation over longer periods of time when treated with ligand than does the wild-type receptor.

ERK1/2 is specifically required for manifestation of constitutive receptor activity

In order to determine the functional relevance of ERK activity in the ligand-independent activation of PFR3^{K650E}, chemical inhibition of ERK with U0126 (45), a selective inhibitor of the MEK1/2 kinases responsible for phosphorylation of ERK (46,47) was performed (Fig. 2B). A dose response curve of the inhibitor between 1 and 30 μ M U0126 was performed on PFR3^{K650E} cell lines treated with inducer in the presence or absence of PDGF (30 ng/ml). In PFR3^{K650E} expressing cells, ~28% of induced cells and 60% of cells induced and treated with PDGF for 15 min showed neurite outgrowth. At 30 μ M U0126, an 80% inhibition of this neurite outgrowth was observed in mutant cells after either induction or in the presence of PDGF (Fig. 2B). As no ligand-independent neurite outgrowth was observed in the presence of the wild-type PFR3 receptor, the inhibitor had no effect (data not shown). FGFR3 levels were not affected by U0126 by western blot analysis (data not shown). In order to confirm that U0126 was acting through inhibition of

ERK1/2 phosphorylation, parallel cultures treated under the same conditions were used for western blot analysis and phosphorylation of ERK1/2 was found to be inhibited by >90% in the presence of 20 μ M U0126 (Fig. 2C). The requirement for ERK1/2 activation and the effect of the MEK inhibitors are specific to the mutant phenotype as neither differential tyrosine phosphorylation of MAPK p38 was observed (Fig. 2A) nor did chemical inhibition of p38 activity by SB202190 (48) have any effect upon neurite outgrowth (data not shown). Therefore, these results support the involvement of ERK signaling pathway activation in the ligand-independent neurite outgrowth phenotype in PFR3^{K650E} pathway.

STAT1 and 3 are activated in a ligand-independent manner in PFR3^{K650E} mutant expressing cells

On the basis of numerous reports in the literature that STAT1 activation is associated with mutant FGFR3 phenotypes and on our gene expression profiling results showing increased p21^{WAF1/CIP1} levels in PFR3^{K650E} expressing PC12 cells, STAT1 phosphorylation was first tested in 293 and PC12 cells transiently expressing either the full length FGFR3 or FGFR3^{K650E} as well as chimeric PFR3 and PFR3^{K650E} to verify the intrinsic capability of these constructs to activate the STAT1 pathway. In both transient assays (data not shown) and subsequent assays in the MT-inducible PC12 cells (Fig. 3A), only in the presence of the K650E mutation is STAT1 tyrosine phosphorylation of a 91 kDa (p91) form observed. An 84 kDa (p84) form of STAT1 is generated by alternative splicing and can be phosphorylated on the same single tyrosine residue as the longer form; however, this splice form has no known activity (49). No differential phosphorylation of the STAT1 serine residue was observed (Fig. 3B). In addition to STAT1 phosphorylation, ligand-independent tyrosine and to some extent serine phosphorylation of STAT3 is observed in PFR3^{K650E} expressing cells (Fig. 3C and data not shown). In contrast, no corresponding differential phosphorylation of STAT5 was found relative to STAT5 protein levels (Fig. 3D). A report by Lievens and Liboi (38) showed STAT1 activation in transient transfection of 293 cells with FGFR3^{K650E}; however, in cells stably transfected with FGFR3^{K650E}, the ability to activate STAT1 was lost. For this reason, an inducible system was used here because high levels of constitutive receptor activity can lead to terminal differentiation and therefore prevent antibiotic selection of relevant cell types expressing adequate receptor levels.

Inhibition of STAT1 and 3 by acute knockdown (siRNA) does not alter ligand-independent K650E associated phenotypes or signaling

Inhibitor studies suggest that ERK activation is likely involved in the effects of constitutive activation of FGFR3^{K650E}. In order to test the implication of STAT activation, a JAK2 inhibitor (AG490) which should inhibit upstream of STAT1 if STAT activation is mediated by the JAK-STAT pathway, was tested for its effect upon neurite outgrowth. This inhibitor shows effects directly opposite to that expected, e.g. an increase in neurite outgrowth was observed. However, this

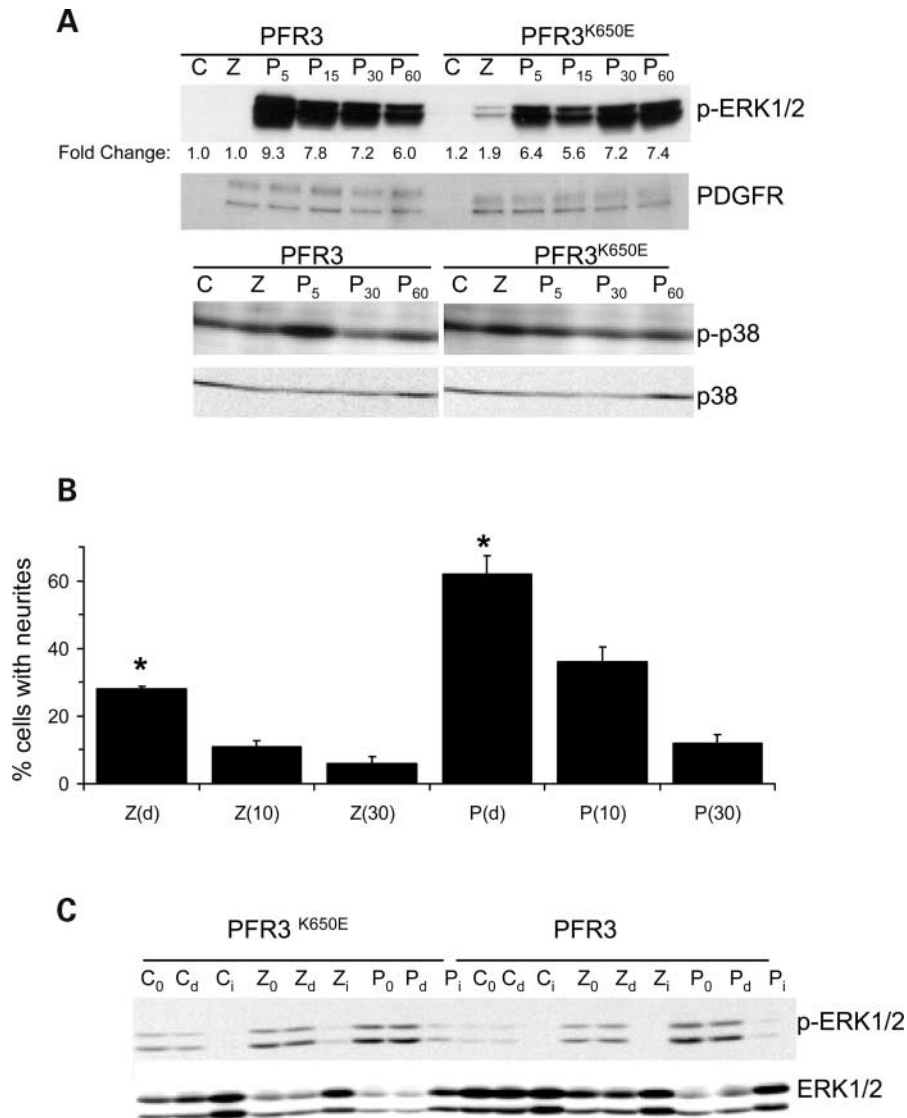


Figure 2. ERK1/2 activation is critical for ligand-independent neurite outgrowth phenotype in PFR3^{K650E} cells. (A) Sustained ERK1/2 activation in PFR3^{K650E} cells. Cells were induced for 24 h followed by treatment with PDGF for the indicated times and described in Materials and Methods. In the PFR3 cell, activated ERK1/2 (i.e. p-ERK1/2) decreases over the 1 h treatment with ligand while in PFR3^{K650E} p-ERK1/2 levels remain high. A low level of ligand-independent ERK activation is observed in PFR3^{K650E} cells (see Z) as well as a low level of p-ERK1/2 even before induction due to some leakiness of the MT promoter (see faint bands in C). ImageJ (version 1.33U) was used to measure the intensity of each band [numbers displayed below each corresponding band are the fold changes from the C lane (uninduced cells)]. The blot was stripped and reprobed with anti-PDGFR antibody to show equal loading between the lanes. The two bands correspond to the p130 and p170 forms of the receptor. In the bottom panels, blots were probed with anti-p38 antibody and stripped and reprobed with antibody against total p38. No difference in activated p38 was observed between PFR3 and PFR3^{K650E} lines. C, uninduced cells; Z, induced cells; P₅–P₆₀, induced cells with a 5–60 min treatment with PDGF. (B) Neurite outgrowth inhibition in PFR3^{K650E} cells after treatment with the MEK1 inhibitor U0126. PFR3^{K650E} cells were plated and the following day cells were induced for 4–5 h. The media was changed to low serum media with inhibitor and without inducer for ≥40 h. PDGF was added for the last 15 min of the inhibitor treatment. Percentage of cells with neurites in PFR3^{K650E} cells is shown. Z, induced; P, PDGF; numbers in parenthesis refer to the concentration of U0126 added in micromolar or d for DMSO control added at an amount equivalent to added U0126 volume. Bars represent the mean ± SE (*, *P* < 0.001, paired *t*-test). Experiments were performed in duplicate at least three times. A representative experiment is shown. (C) Western blots showing inhibition of ERK1/2 activation by U0126. Experiments were performed as described in (B) except the inhibitor treatment was only 20 h. Blot was stripped and reprobed with antibody against total ERK1/2. C, uninduced cells; Z, induced; P, PDGF addition. For the subscripts: 0, nothing added; d, DMSO added (see before); i, 20 μM U0126.

inhibitor has been reported to have effects other than upon JAK/STAT signaling (50). Because of the lack of specificity inherent in AG490 treatment, genetic approaches to determine the functional relevance of STAT1 activation were performed. Transient transfection of dominant negative *stat1* (provided by Y. Wu) was first used to suppress STAT1 activation and no

effect upon neurite outgrowth or ERK phosphorylation was observed (data not shown). To inhibit STAT1 activity in a highly specific manner, interfering RNAs (siRNAs) were used to silence STAT1 activity through a reduction of STAT1 protein levels. Several siRNAs were tested and Dharmacon siRNAs found to silence the most effectively

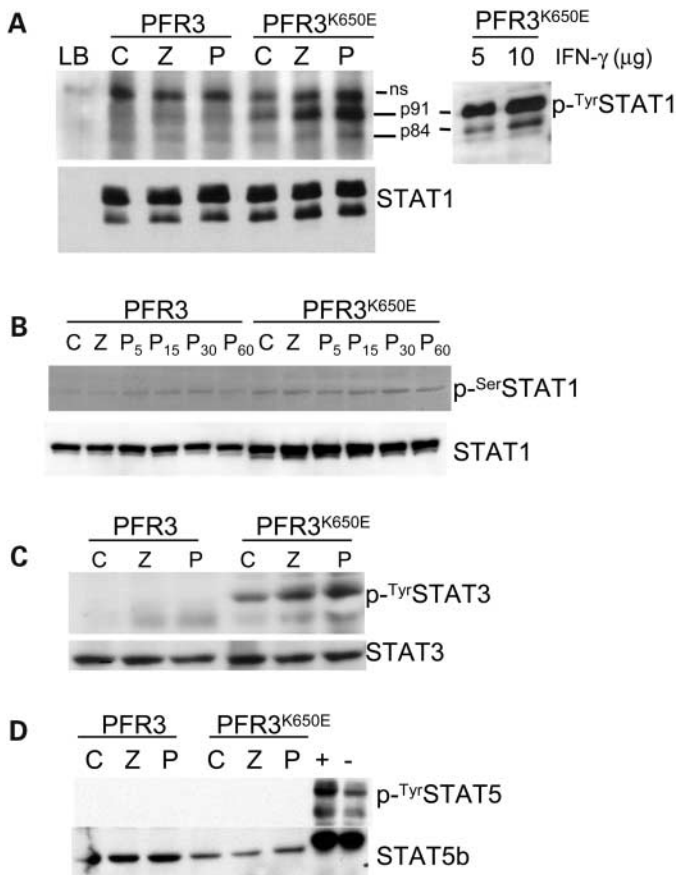


Figure 3. STAT1 and 3 activities are ligand-independent in PFR3^{K650E} cells. (A) Western blot with anti-phospho-Tyr⁷⁰¹STAT1 antibody. The p91 and p84 bands are two isoforms of the STAT1 protein. In the PFR3^{K650E} cells, the p91 form of STAT1 becomes phosphorylated (even in the absence of induction), whereas in PFR3 cells, even the addition of PDGF does not lead to detectable phosphorylation of the p91. Interferon (IFN)- γ treated PFR3^{K650E} cells are used as a control for tyrosine phosphorylation and activation of STAT1. Identical results are obtained for PFR3 expressing lines. Activation by (IFN)- γ is greater than that observed for ligand-independent activation of PFR3. C, uninduced cells; Z, induced for 22 h; P, induced with the addition of PDGF for the final 30 min. LB, NP-40 lysis buffer; ns, non-specific band. (B) Time course of phospho-Ser⁷²⁷STAT1 activation following PDGF addition. No difference in serine phosphorylation of STAT1 was observed between PFR3^{K650E} and PFR3 cells. C, uninduced cells; Z, induced for 22 h; P, induced with the addition of PDGF for indicated times (subscript). (C) Phospho-Tyr⁷⁰⁵STAT3. STAT3 becomes tyrosine phosphorylated even in the absence of inducer in PFR3^{K650E} but not in PFR3 cells. A total of 50 μ g protein was loaded per lane. (D) Phospho-Tyr⁶⁹⁴STAT5. No activated STAT5 was detected in the PFR3^{K650E} and PFR3 cells. + and - lanes are EGF treated and untreated human A431 cells (Cell Signaling Technology), respectively (controls). For (C and D) labels are the same as in (A). For (A–D) lower panels show blots that were stripped and reprobed with antibodies detecting total STAT1 (A and B), STAT3 (C) and STAT5 (D).

with the highest specificity as assessed by western analysis of STAT1, STAT3 and STAT5b protein and noting that STAT1 is specifically reduced. Silencing of STAT1 was observed as early as 48 h post-transfection and persisted up to 96 h post-transfection (Fig. 4A).

When STAT1 silencing was tested for effects upon PFR3^{K650E}-mediated ligand-independent neuritogenesis, no effect on ligand-independent neurite outgrowth was observed

in PFR3^{K650E} expressing cells transfected with rat STAT1 siRNA at 1 and 2 days post-induction. In addition to neurite outgrowth, expression of a putative STAT1 target, e.g. p21^{WAF1/CIP1}, and activation of signaling pathways implicated in the growth arrest phenotype, e.g. ERK1/2, were assessed in the presence of STAT1 siRNA. No alteration in either phospho-ERK1/2 or p21^{WAF1/CIP1} protein levels was observed in STAT1 siRNA transfected PFR3^{K650E} cells (Fig. 4B).

To determine whether STAT3 activation is critical to ligand-independent neurite outgrowth as was reported by Hart *et al.* (31), siRNAs for STAT3 were also developed and tested. As with STAT1 siRNA, STAT3 was specifically silenced; however, no corresponding alteration in neurite outgrowth (described subsequently) or ERK1/2 phosphorylation was observed (Fig. 4C). Finally, to determine whether STAT1 and 3 are compensating for the reduction of the other, thereby masking any effects upon p21^{WAF1/CIP1} or phospho-ERK1/2, siRNAs to both STAT1 and STAT3 were co-transfected and evaluated. We did not see a combined effect of STAT1+3 inhibition on neurite outgrowth (Table 1) (ANOVA single factor among no siRNA, STAT1 siRNA, STAT3 siRNA and STAT1+3 siRNA, $P = 0.52$). Figure 4D shows that STAT1 and 3 levels are drastically reduced, whereas no effects upon phospho-ERK1/2 or p21^{WAF1/CIP1} levels are observed. Similar results were obtained when STAT activity was inhibited when a dominant negative *stat3* was transfected (provided by Y. Wu) singly or in combination with dominant negative *stat1* (data not shown).

DISCUSSION

Using PC12 cells, we have characterized the effects of a stably transfected and inducible chimeric PDGFR–FGFR3 containing a mutation that causes neonatal lethal TD. We show that the expression of this mutation causes sustained activation of the ERK pathway, required for the ligand-independent differentiation observed upon expression of the K650E containing FGFR3 receptor. We also provide evidence that ligand-independent activation of the receptor causes STAT1 and STAT3 but not STAT5 activation. STAT1 and/or 3 activation is not required, however, for FGFR3 mutant induced neurite outgrowth, ERK activation or p21^{WAF1/CIP1} induction as evidenced by silencing of STAT1 and/or STAT3.

FGFR3 is expressed in proliferating and prehypertrophic chondrocytes of developing cartilage. Chondrocytes first proliferate, then exit the cell cycle, transit to early hypertrophy and undergo hypertrophic differentiation. Both proliferation and complete hypertrophy are inhibited by skeletal dysplasia mutations in FGFR3, whereas the transition to or initiation of early hypertrophy (differentiation) are premature (30,42,51). FGFR3 signaling is unique in both PC12 cells and chondrocytes in that both cell types are called upon to cease dividing and differentiate upon activation by FGFs: into neurites in PC12 cells and into hypertrophic chondrocytes in chondrocyte. As no chondrocyte line exists that inducibly expresses the mutant FGFR3 receptor, it has been difficult to assess the immediate effects of this mutation and evaluate the functional significance of ERK and STAT signaling

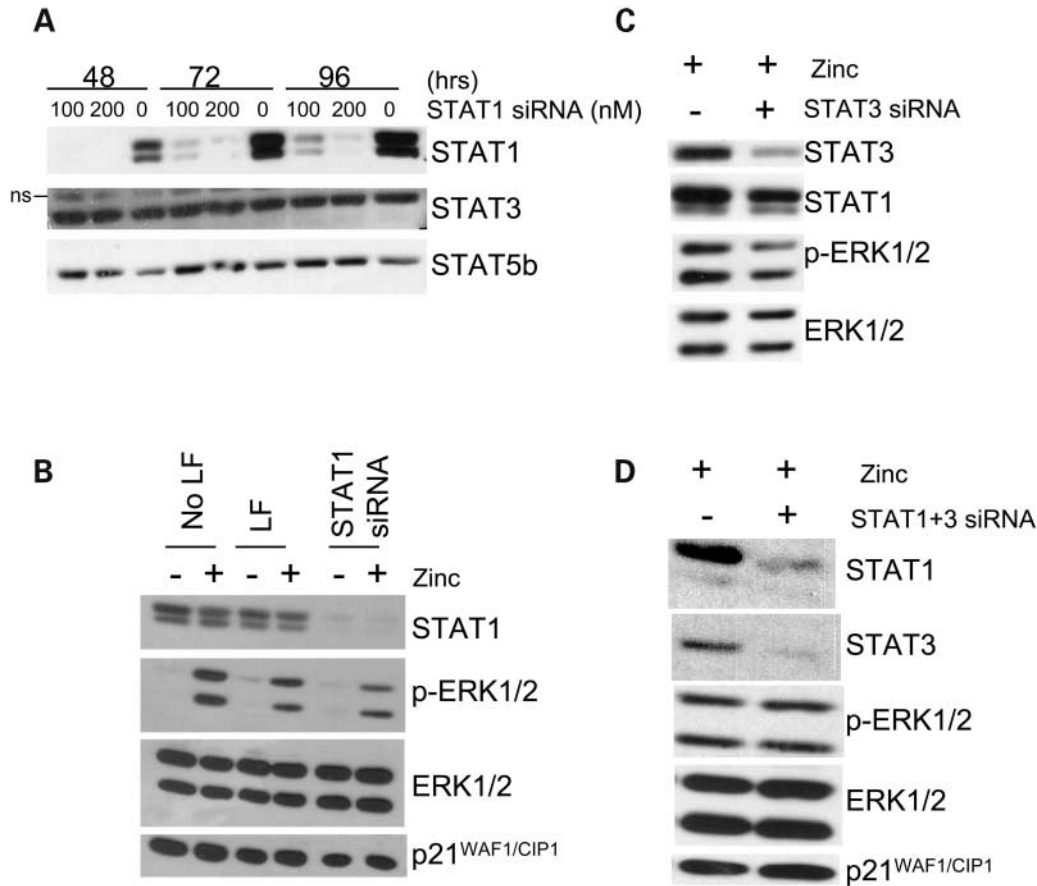


Figure 4. Silencing of STAT1 and STAT3 has no effect on the activation of ERK or p21^{WAF1/CIP1} protein levels in PFR3^{K650E} cells. **(A)** Time and concentration dependence of STAT1 siRNA treatment. Cells were transfected with 0, 100 or 200 nM STAT1 siRNA and harvested at 48, 72 and 96 h post-transfection. Western blot analysis was performed with the indicated antibodies. **(B)** STAT1 siRNA. Cells without Lipofectamine 2000 (no LF), with Lipofectamine 2000 (LF) or with 100 nM STAT1 siRNA were incubated for 16–18 h and the media was changed to fresh complete media. Two days post-transfection cells were induced (+) or left uninduced (-) for an additional 24 h. Shown are western blots with the indicated antibodies. To rule out the possibility that the transfection reagent Lipofectamine 2000 (LF) might affect the outcome of our experiment, PFR3^{K650E} cell lysates were incubated with and without LF and no effect with LF on the protein level of the STAT1, p-ERK1/2, ERK1/2 or p21^{WAF1/CIP1} as observed. **(C)** STAT3 siRNA. Experiments were performed as described in (B) except using 100 nM STAT3 siRNA. Shown are western blots with the indicated antibodies. **(D)** STAT1+3 siRNAs. Experiments were performed as described in (B). A concentration of 100 nM was used for each siRNA separately or in combination. Shown are western blots with the indicated antibodies.

upon hypertrophic differentiation through biochemical manipulations in cells. Wild-type RCS chondrocytes, which primarily express the type 3 form of the FGFRs, can be induced to undergo growth arrest upon the addition of FGF2 and the ERK signaling pathway appears to be essential for this growth arrest (40), similar to the results presented here showing that ERK phosphorylation is required for the manifestation of ligand-independent initiation of differentiation and ligand-dependent neurite outgrowth. Although signal transduction pathways in chondrocytes may not be completely modeled in PC12 cells, each cell type is influenced by FGFR3 signaling in a similar manner and parallel molecular events, such as ERK and STAT activation and p21 induction, occurs in each cell type upon expression of the mutant FGFR3 receptor. Although molecular mechanisms will ultimately have to be resolved in chondrocytes, PC12 cells allow one to examine molecular processes triggered by normal and mutant receptors on a comparative basis and effects upon premature initiation of differentiation in PC12

cells can be reasonably extrapolated to signaling activities that result in premature initiation of differentiation of the growth plate in mouse models and human cartilage.

Following the identification of FGFR3 as the genetic locus for ACH, a number of studies were carried out to examine the effects of the several identified mutations upon the signaling responses of this receptor (31–35). These adopted a number of approaches and paradigms and lead to the general conclusion that the ACH subgroup of skeletal dysplasias was due to gain of function mutations that resulted in graded constitutive activation of the receptor, corresponding to the severity of the mutant phenotype (52). Expression of the activated FGFR3 mutants in mice reproduces many features of the dwarf phenotype of the human skeletal disorders, whereas lack of FGFR3 in mice causes skeletal overgrowth (12,14–19). FGFR3, thus, appears to be a negative regulator of chondrocyte proliferation and accelerates the transition to hypertrophy while inhibiting complete hypertrophic differentiation (20,41,43,53,54).

Table 1. Silencing of STAT1 and STAT3 does not modify ligand-independent neurite outgrowth of PFR3^{K650E} cells

Condition	Mean \pm SD (%)
No siRNA	55.8 \pm 6.3
STAT1 siRNA	68.3 \pm 1.5
STAT3 siRNA	53.7 \pm 9.1
STAT1+3 siRNA	59.7 \pm 22.4

Cells were transfected with STAT1 or STAT3 siRNAs singly or STAT1+3 siRNAs in combination (100 nM) and compared with lipofectamine control. The cells were examined for the presence of neurites 24 h post-induction as described in Materials and Methods. Responsive cells were defined as those bearing neurites at least two cell diameters in length. This shows mean percentage of transfected cells with neurites \pm standard deviation (SD). Three microscopic fields were examined. An eGFP-N1 plasmid was co-transfected with the siRNAs to mark the transfected cells. Using single factor ANOVA statistical analysis, no significant differences were observed among the groups ($P = 0.52$).

Similar to FGFR3 activating mutations, overexpression of FGF2 in transgenic mice leads to a type of dwarfism (55) which is improved when these mice were crossed with STAT1 knockout mice (56). FGF1 treatment of murine bone rudiment cultures results in decreased chondrocyte proliferation, but this effect is not observed in STAT1 knockout mice (25). However, treatment of limb explants from p21^{WAF1/CIP1} knockout mice with FGF2 only partially corrected the shortening seen in controls (57), suggesting this is only one of several possible targets. Although it is known that p21^{WAF1/CIP1} is a STAT target gene, neither STAT1 nor 3 activation is responsible for induction of p21^{WAF1/CIP1} expression in the PC12 cells, even though these STATs are phosphorylated. Among the possible mechanisms that may lead to increased p21^{WAF1/CIP1} levels, ERK activation itself may be responsible for this induction (58,59); the precise mechanism is under further investigation. Recently, Legeai-Mallet *et al.* (24) showed that in tibial cartilage from ACH and TD fetuses obtained at autopsy, overexpression of FGFR3, STAT1, STAT5 and p21^{WAF1/CIP1} correlates with phenotypic severity and defective chondrocyte differentiation in FGFR3-related chondrodysplasias. The authors conclude that defective differentiation of chondrocytes in human patients is likely to be the major cause of reduced bone growth in FGFR3-related skeletal dysplasias. Activation of STAT3 has been assessed to date only in 293T cells expressing TD-like derivatives of FGFR3 (31). The importance of STAT1 in the pathophysiology of FGFR3 mutations has recently been challenged. Although genetic evidence has identified STAT1 as a mediator of the FGF-induced growth arrest of chondrocytes both *in vitro* and *in vivo*, the mechanism by which STAT1 participates in this process is still unclear and may not require that it functions as a transcriptional regulator or that its role is specific to a particular aspect of pathology.

In PC12 cells, Wu *et al.* (60) showed that although STAT3 and to a lesser extent STAT1 is phosphorylated upon stimulation with FGF2, this apparent activation is not sufficient or required for neurite outgrowth in native PC12 cells. In addition, data from Raucchi *et al.* (23) show that interferon- γ , a potent inducer of STAT1 transcriptional activation, does

not induce growth arrest in RCS cells. Neither STAT1 nor p21^{WAF1/CIP1} knockout mice (61) have dwarfism, but the STAT1 knockout mice have an expanded proliferative zone and show accelerated growth, ending up normal in length (56). Murakami *et al.* (30) recently showed that although loss of STAT1 restored the reduced chondrocyte proliferation in mice expressing an ACH mutant of FGFR3, it did not rescue the reduced hypertrophic zone, the delay in the formation of secondary ossification centers, and the ACH-like phenotype. The authors suggested a model in which FGFR3 signaling inhibits bone growth by inhibiting chondrocyte differentiation through the ERK pathway and by inhibiting chondrocyte proliferation through STAT1.

In accordance with our observations that sustained ERK activity is critical for ligand-independent phenotypes, expression of a constitutively active mutant of MEK1 in chondrocytes of FGFR3-deficient mice inhibited skeletal overgrowth (30), strongly suggesting that the regulation of bone growth by FGFR3 is mediated at least in part by the MEK/ERK pathway. In addition, inhibition of the MAPK pathway through the treatment of ACH mice with C-type natriuretic peptide prevented the shortening of achondroplastic bones, while having no effect on the STAT1 pathway (41). Taken together, these results suggest that the effects of the MAPK pathway on growth-plate chondrocytes are independent of STAT1, arguing against crosstalk between the two pathways that may influence phenotypic outcomes.

While STAT1 does not appear to influence the reduced hypertrophic zone or other phenotypes in ACH mice (30), the functional role of STAT1 has not been previously assessed in TD-expressing cells nor has the role of STAT3 in any mutant FGFR3 system. In this report, we systematically tested the requirement for both STAT1 and 3 activities singly and in combination using specific siRNAs in a system that models the differentiation component of FGFR3 signaling. The ligand-independent activation of FGFR3 in the presence of a constitutive activating mutation is a complex event that requires the intervention of several proteins. Although we cannot rule out that STATs are having effects on these cells that we are not measuring, the events attributed to the activation of the STAT pathway in chondrocytes do not appear to be required for the phenotypic readouts assessed here. Among the signaling pathways downstream of FGFR3, sustained ERK activation plays an essential role in PC12 cell differentiation. STAT1 and 3 activities, previously thought to be potential modulators of p21 induction and premature growth arrest or initiation of differentiation that can occur in the absence of ligand, are not required.

MATERIALS AND METHODS

Propagation of PC12 cell lines

Construction of chimeric receptors containing PFR3 (wild-type) or PFR3^{K650E} (mutant) receptors under the control of the bovine MT promoter in pMT-CB6+ and generation of inducible PC12 (rat pheochromocytoma) cell lines have been described previously (33). Three clones were selected for both the wild-type and mutant lines which had similar receptor expression levels and receptor numbers per cell (determined

independently by COR Therapeutics, San Francisco, CA, USA). Cells were maintained in T-150 tissue culture flasks in complete medium [DMEM (Invitrogen), 5% platelet-poor plasma horse serum (PPHS) (Sigma), 2.5% plasma-derived fetal bovine serum (Cocalbiologicals)] at 37°C, 5% CO₂. For experiments, cells were plated on collagen coated plates at 5×10^5 cells per well (for six-well plates) and expression of chimeric receptors was induced by changing media to low serum media (DMEM, 1% PPPHS) and addition of 80 μ M Zn²⁺ (zinc sulfate) as indicated. Where indicated, human PDGF-BB ligand (PDGF, Austral Biologicals) was added at 30 ng/ml for the indicated times. All experiments were performed at least three times.

RNA sample preparation and microarrays

For gene expression profiling studies three clones of each line (described earlier), PFR3 or PFR3^{K650E} cells were plated in 150 mm plates and the following day induced for 24 h. Total RNA was isolated separately for each clone using Trizol Reagent according to the manufacturer (Invitrogen). RNA was quantitated spectrophotometrically and integrity tested by capillary electrophoresis (Agilent 2100 Bioanalyzer). Equal amounts of RNA from each clone of mutant and wild-type lines were pooled and a total of 25 μ g total RNA was used to generate target cRNAs for hybridization to Affymetrix Rat Genome U34A oligonucleotide arrays (UCI DNA Array Core Facility). Three separate cell growths were done for each clone and cRNAs were synthesized from the pooled RNAs separately and triplicate GeneChips were performed. Gene expression profiling is described in Supplementary Material.

Northern blot analysis

For northern blot analysis, total RNA was isolated from cells grown on 150 mm plates using Qiagen RNeasy Midi Kit. RNA was formaldehyde denatured as previously described and 50 μ g of total RNA was used per lane (62). Hybridization and wash conditions were as described by Clontech Laboratories (Catalog no. PR63271) and blots were hybridized for 18–24 h at 42°C. Signals were detected by exposing to a phosphor screen, scanning with a Molecular Dynamics Phosphorimager and bands were quantitated using ImageQuant software. The p21^{WAF1/CIP1} probe was generated by an *Eco*RI/*Hind*III restriction digest of pCRW0.8 (kindly provided by Dr Bert Vogelstein, John Hopkins University School of Medicine). Other probes were generated by RT-PCR and sequence verified. The primers were aldehyde dehydrogenase [TAGATGATGTGATCAAGAGAGCAAA] (forward) and [ATTCCCAAATAATTACAGACCAACA] (reverse) (GenBank accession no. AF001898); Cyclin D2 [CTTCAGCAGGACGAGGAAGT] (forward) and [CTTCTC ATTTCTGATTCTTCTTGG] (reverse) (GenBank accession no. L09752); Early growth response1 (egr1) [ACTAGAAC ATCAAGTTGGCTGAAA] (forward) and [TTGTTTAA GCAAACACAAGTACGAA] (reverse) (GenBank accession no. NM_012551) and EF1 α [GACTTCATCAAGAACAT GAT] (forward) and [GTGCCAATGCCGCAAT] (reverse) (GenBank accession no. X13661). Probes were labeled by

random priming using the method of Feinberg and Vogelstein (63). Blots were stripped and reprobed with EF1 α to normalize for variations in loading.

Antibodies and inhibitors

The following antibodies were used: phospho-Tyr⁷⁰⁵STAT3, phospho-Tyr⁶⁹⁴STAT5, phospho-p38, p38, phospho-ERK1/2 and ERK1/2 were from Cell Signaling Technology, phospho-Ser⁷²⁷ STAT1 and phospho-Tyr⁷⁰¹STAT1 from Upstate Technology. STAT1, STAT3, STAT5b and p21^{WAF1/CIP1} were from Santa Cruz Biotechnology and PDGFR was from Austral Biologicals. For the inhibitor studies: U0126 (Cell Signaling Technology), SB202190 and AG490 (Calbiochem) inhibitors were resuspended in DMSO.

Harvesting cells, immunoprecipitation and western blot analysis

Cells were routinely harvested by placing plates on ice, aspirating media and direct lysis using NP-40 lysis buffer (50 mM Hepes, pH 7.8, 0.5% Nonidet P-40, 100 mM sodium fluoride, 10 mM sodium pyrophosphate, 0.5 mM sodium orthovanadate, 10 μ g/ml each of aprotinin and leupeptin and 1 mM phenylmethylsulfonyl fluoride). Lysates were centrifuged at 14 000g, 4°C and the supernatants were frozen at –80°C. For western blot analysis, 20 μ g soluble protein was loaded per lane (unless otherwise indicated) onto 10% SDS-PAGE. For phospho-STAT1 immunoprecipitation, cells were harvested as described previously, pellets were lysed with NP-40 lysis buffer and 1 mg protein was incubated with 3 μ g anti-STAT1 antibody at 4°C overnight followed by 1 h incubation with protein A-Agarose (Santa Cruz Biotechnology). Immunoprecipitates were washed 3 \times in NP-40 lysis buffer and 1 \times with 20 mM Tris-HCl, pH 7.3. ImageJ (Version 1.33U), a public domain Java image processing program inspired by NIH Image was used to measure the intensity of each band and fold changes calculated from these data.

Neurite outgrowth assay

For neurite outgrowth assays, PC12 cells were plated in six-well plates at a low density of 1×10^5 cells/well. After 16–24 h, cells were rinsed with PBS and changed to low serum media with inducer for 1 day followed by treatment with PDGF for 5–60 min. Cells were examined for the presence of neurites 24 or 48 h post-induction. Responsive cells were defined as those bearing neurites at least two cell diameters in length.

siRNA assay

siRNAs were designed by Dharmacon (Lafayette, CA, USA). Dharmacon SMARTpool uses an algorithm to combine four or more SMART selected siRNA duplexes in a single pool. PFR3^{K650E} cells were plated in six-well plates and the following day transfected with 100 nM Dharmacon SMARTpool siRNA and 160 ng pEGFP-N1 vector (to monitor transfection efficiency, BD Biosciences) using Lipofectamine 2000 (Invitrogen). Two days later the media was changed and

cells were induced in low serum for 1 day (i.e. 3 days post-transfection) prior to harvesting. Neurite outgrowth assays were performed at 1 day post-induction. For time courses and concentration titration experiments, uninduced cells were transfected with 0, 100 and 200 nM STAT1 siRNA and cells were harvested at 48, 72 and 96 h post-transfection.

SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS

This study was supported by Yang Sheng Tang, USA. We would like to thank Drs William Wilcox, Yvonne Wu and Pavel Krejci for helpful discussions and Dr Wu for the dominant negative *stat* constructs.

Conflict of Interest statement. None declared.

REFERENCES

- Shiang, R., Thompson, L.M., Zhu, Y.-Z., Church, D.M., Fielder, T.J., Bocian, M., Winokur, S.T. and Wasmuth, J.J. (1994) Mutations in the transmembrane domain of FGFR3 cause the most common genetic form of dwarfism, achondroplasia. *Cell*, **78**, 335–342.
- Rousseau, F., Bonaventure, J., Legeai-Mallet, L., Pelet, A., Rozet, J.-M., Maroteaux, P., Le Merrer, M. and Munnich, A. (1994) Mutations in the gene encoding fibroblast growth factor receptor-3 in achondroplasia. *Nature*, **371**, 252–254.
- Bellus, G.A., Gaudenz, K., Zackai, E.H., Clarke, L.A., Szabo, J., Francomano, C.A. and Muenke, M. (1996) Identical mutations in three different fibroblast growth factor receptor genes in autosomal dominant craniosynostosis syndromes. *Nat. Genet.*, **14**, 174–176.
- Tavormina, P.L., Shiang, R., Thompson, L.M., Zhu, Y.-Z., Wilkin, D.J., Lachman, R.S., Wilcox, W.R., Rimoin, D.L., Cohn, D.H. and Wasmuth, J.J. (1995) Thanatophoric dysplasia (types I and II) caused by distinct mutations in fibroblast growth factor receptor 3. *Nat. Genet.*, **9**, 321–328.
- Meyer, A.N., Gastwirt, R.F., Schlaepfer, D.D. and Donoghue, D.J. (2004) The cytoplasmic tyrosine kinase Pyk2 as a novel effector of fibroblast growth factor receptor 3 activation. *J. Biol. Chem.*, **279**, 28450–28357.
- Boilly, B., Vercoutter-Edouart, A.S., Hondermarck, H., Nurcombe, V. and Le Bourhis, X. (2000) FGF signals for cell proliferation and migration through different pathways. *Cytokine Growth Factor Rev.*, **11**, 295–302.
- Kreuger, J., Jemth, P., Sanders-Lindberg, E., Eliahu, L., Ron, D., Basilico, C., Salmivirta, M. and Lindahl, U. (2005) Fibroblast growth factors share binding sites in heparan sulfate. *Biochem. J.*, published ahead of print (16 March 2005). doi: 10.1042/BJ20042129.
- Ornitz, D.M. (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays*, **22**, 108–112.
- Murakami, S., Kan, M., McKeehan, W.L. and de Crombrugge, B. (2000) Up-regulation of the chondrogenic *Sox9* gene by fibroblast growth factors is mediated by the mitogen-activated protein kinase pathway. *Proc. Natl Acad. Sci. USA*, **97**, 1113–1118.
- Peters, K., Ornitz, D., Werner, S. and Williams, L. (1994) Unique expression pattern of the FGF receptor 3 gene during mouse organogenesis. *Dev. Biol.*, **155**, 423–430.
- Delezoide, A.L., Benoist-Lasselin, C., Legeai-Mallet, L., Le Merrer, M., Munnich, A., Vekemans, M. and Bonaventure, J. (1998) Spatio-temporal expression of FGFR 1, 2 and 3 genes during human embryo-fetal ossification. *Mech. Dev.*, **77**, 19–30.
- Naski, M.C. and Ornitz, D.M. (1998) FGF signaling in skeletal development. *Front. Biosci.*, **3**, D781–D794.
- Ohbayashi, N., Shibayama, M., Kurotaki, Y., Imanishi, M., Fujimori, T., Itoh, N. and Takada, S. (2002) FGF18 is required for normal cell proliferation and differentiation during osteogenesis and chondrogenesis. *Genes Dev.*, **16**, 870–879.
- Li, C., Chen, L., Iwata, T., Kitagawa, M., Fu, X.Y. and Deng, C.X. (1999) A Lys644Glu substitution in fibroblast growth factor receptor 3 (FGFR3) causes dwarfism in mice by activation of STATs and ink4 cell cycle inhibitors. *Hum. Mol. Genet.*, **8**, 35–44.
- Wang, Y., Spatz, M.K., Kannan, K., Hayk, H., Avivi, A., Gorivodsky, M., Pines, M., Yayon, A., Lonai, P. and Givol, D. (1999) A mouse model for achondroplasia produced by targeting fibroblast growth factor receptor 3. *Proc. Natl Acad. Sci. USA*, **96**, 4455–4460.
- Iwata, T., Li, C.L., Deng, C.X. and Francomano, C.A. (2001) Highly activated Fgfr3 with the K644M mutation causes prolonged survival in severe dwarf mice. *Hum. Mol. Genet.*, **10**, 1255–1264.
- Iwata, T., Chen, L., Li, C., Ovchinnikov, D.A., Behringer, R.R., Francomano, C.A. and Deng, C.X. (2000) A neonatal lethal mutation in FGFR3 uncouples proliferation and differentiation of growth plate chondrocytes in embryos. *Hum. Mol. Genet.*, **9**, 1603–1613.
- Segev, O., Chumakov, I., Nevo, Z., Givol, D., Madar-Shapiro, L., Sheinin, Y., Weinreb, M. and Yayon, A. (2000) Restrained chondrocyte proliferation and maturation with abnormal growth plate vascularization and ossification in human FGFR-3(G380R) transgenic mice. *Hum. Mol. Genet.*, **9**, 249–258.
- Chen, L., Li, C., Qiao, W., Xu, X. and Deng, C. (2001) A Ser(365) → Cys mutation of fibroblast growth factor receptor 3 in mouse downregulates Ihh/PTHrP signals and causes severe achondroplasia. *Hum. Mol. Genet.*, **10**, 457–465.
- Colvin, J.S., Bohne, B.A., Harding, G.W., McEwen, D.G. and Ornitz, D.M. (1996) Skeletal overgrowth and deafness in mice lacking fibroblast growth factor receptor 3. *Nat. Genet.*, **12**, 290–397.
- Deng, C., Wynshaw-Boris, A., Zhou, F., Kuo, A. and Leder, P. (1996) Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell*, **84**, 911–921.
- Su, W.-C.S., Kitagawa, M., Xue, N., Xie, B., Garofalo, S., Cho, J., Deng, C., Horton, W.A. and Fu, X.-Y. (1997) Activation of Stat1 by mutant fibroblast growth-factor receptor in thanatophoric dysplasia type II dwarfism. *Nature*, **386**, 288–292.
- Rauci, A., Laplantine, E., Mansukhani, A. and Basilico, C. (2004) Activation of the ERK1/2 and p38 mitogen-activated protein kinase pathways mediates fibroblast growth factor-induced growth arrest of chondrocytes. *J. Biol. Chem.*, **279**, 1747–1756.
- Legeai-Mallet, L., Benoist-Lasselin, C., Munnich, A. and Bonaventure, J. (2004) Overexpression of FGFR3, Stat1, Stat5 and p21Cip1 correlates with phenotypic severity and defective chondrocyte differentiation in FGFR3-related chondrodysplasias. *Bone*, **34**, 26–36.
- Sahni, M., Ambrosetti, D.C., Mansukhani, A., Gertner, R., Levy, D. and Basilico, C. (1999) FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev.*, **13**, 1361–1366.
- Debiais, F., Lefevre, G., Lemonnier, J., Le Mee, S., Lasmoles, F., Mascarelli, F. and Marie, P.J. (2004) Fibroblast growth factor-2 induces osteoblast survival through a phosphatidylinositol 3-kinase-dependent, -beta-catenin-independent signaling pathway. *Exp. Cell Res.*, **297**, 235–246.
- Ronchetti, D., Greco, A., Compasso, S., Colombo, G., Dell'Era, P., Otsuki, T., Lombardi, L. and Neri, A. (2001) Deregulated FGFR3 mutants in multiple myeloma cell lines with t(4;14): comparative analysis of Y373C, K650E and the novel G384D mutations. *Oncogene*, **20**, 3553–3562.
- Kong, M., Wang, C.S. and Donoghue, D.J. (2002) Interaction of fibroblast growth factor receptor 3 and the adapter protein SH2-B. A role in STAT5 activation. *J. Biol. Chem.*, **277**, 15962–15970.
- Shimoaka, T., Ogasawara, T., Yonamine, A., Chikazu, D., Kawano, H., Nakamura, K., Itoh, N. and Kawaguchi, H. (2002) Regulation of osteoblast, chondrocyte, and osteoclast functions by fibroblast growth factor (FGF)-18 in comparison with FGF-2 and FGF-10. *J. Biol. Chem.*, **277**, 7493–7500.
- Murakami, S., Balmes, G., McKinney, S., Zhang, Z., Givol, D. and de Crombrugge, B. (2004) Constitutive activation of MEK1 in chondrocytes causes Stat1-independent achondroplasia-like dwarfism and rescues the Fgfr3-deficient mouse phenotype. *Genes Dev.*, **18**, 290–305.
- Hart, K.C., Robertson, S.C., Kanemitsu, M.Y., Meyer, A.N., Tynan, J.A. and Donoghue, D.J. (2000) Transformation and Stat activation by derivatives of FGFR1, FGFR3 and FGFR4. *Oncogene*, **19**, 3309–3320.

32. Naski, M.C., Wang, Q., Xu, J. and Ornitz, D.M. (1996) Graded activation of fibroblast growth factor receptor 3 by mutations causing achondroplasia and thanatophoric dysplasia. *Nat. Genet.*, **13**, 233–237.
33. Raffioni, S., Zhu, Y.Z., Bradshaw, R.A. and Thompson, L.M. (1998) Effect of transmembrane and kinase domain mutations on fibroblast growth factor receptor 3 chimera signaling in PC12 cells. A model for the control of receptor tyrosine kinase activation. *J. Biol. Chem.*, **273**, 35250–35259.
34. Webster, M.K. and Donoghue, D.J. (1997) FGFR activation in skeletal disorders: too much of a good thing. *Trends Genet.*, **13**, 178–182.
35. Webster, M.K. and Donoghue, D.J. (1997) Enhanced signaling and morphological transformation by a membrane-localized derivative of the fibroblast growth factor receptor 3 kinase domain. *Mol. Cell. Biol.*, **17**, 5739–5747.
36. Cormier, S., Delezoide, A.L., Benoist-Lassel, C., Legeai-Mallet, L., Bonaventure, J. and Silve, C. (2002) Parathyroid hormone receptor type 1/Indian hedgehog expression is preserved in the growth plate of human fetuses affected with fibroblast growth factor receptor type 3 activating mutations. *Am. J. Pathol.*, **161**, 1325–1335.
37. Legeai-Mallet, L., Benoist-Lassel, C., Delezoide, A.L., Munnich, A. and Bonaventure, J. (1998) Fibroblast growth factor receptor 3 mutations promote apoptosis but do not alter chondrocyte proliferation in thanatophoric dysplasia. *J. Biol. Chem.*, **273**, 13007–13014.
38. Lievens, P.M. and Liboi, E. (2003) The thanatophoric dysplasia type II mutation hampers complete maturation of fibroblast growth factor receptor 3 (FGFR3), which activates signal transducer and activator of transcription 1 (STAT1) from the endoplasmic reticulum. *J. Biol. Chem.*, **278**, 17344–17349.
39. Lievens, P.M., Mutinelli, C., Baynes, D. and Liboi, E. (2004) The kinase activity of fibroblast growth factor receptor 3 with activation loop mutations affects receptor trafficking and signaling. *J. Biol. Chem.*, **279**, 43254–43260.
40. Krejci, P., Bryja, V., Pachernik, J., Hampl, A., Pogue, R., Mekikian, P. and Wilcox, W.R. (2004) FGF2 inhibits proliferation and alters the cartilage-like phenotype of RCS cells. *Exp. Cell Res.*, **297**, 152–164.
41. Yasoda, A., Komatsu, Y., Chusho, H., Miyazawa, T., Ozasa, A., Miura, M., Kurihara, T., Rogi, T., Tanaka, S., Suda, M. *et al.* (2004) Overexpression of CNP in chondrocytes rescues achondroplasia through a MAPK-dependent pathway. *Nat. Med.*, **10**, 80–86.
42. Ornitz, D.M. and Marie, P.J. (2002) FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.*, **16**, 1446–1465.
43. Dailey, L., Laplantine, E., Priore, R. and Basilico, C. (2003) A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation. *J. Cell Biol.*, **161**, 1053–1066.
44. Vaudry, D., Stork, P.J., Lazarovici, P. and Eiden, L.E. (2002) Signaling pathways for PC12 cell differentiation: making the right connections. *Science*, **296**, 1648–1649.
45. Favata, M.F., Horiuchi, K.Y., Manos, E.J., Daulerio, A.J., Stradley, D.A., Feeser, W.S., Van Dyk, D.E., Pitts, W.J., Earl, R.A., Hobbs, F. *et al.* (1998) Identification of a novel inhibitor of mitogen-activated protein kinase kinase. *J. Biol. Chem.*, **273**, 18623–18632.
46. Aletsee, C., Brors, D., Palacios, S., Pak, K., Mullen, L., Dazert, S. and Ryan, A.F. (2002) The effects of laminin-1 on spiral ganglion neurons are dependent on the MEK/ERK signaling pathway and are partially independent of Ras. *Hear. Res.*, **164**, 1–11.
47. Schmitt, J.M., Wayman, G.A., Nozaki, N. and Soderling, T.R. (2004) Calcium activation of ERK mediated by calmodulin kinase I. *J. Biol. Chem.*, **279**, 24064–24072.
48. Cuenda, A., Rouse, J., Doza, Y.N., Meier, R., Cohen, P., Gallagher, T.F., Young, P.R. and Lee, J.C. (1995) SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.*, **364**, 229–233.
49. Walter, M.J., Look, D.C., Tidwell, R.M., Roswit, W.T. and Holtzman, M.J. (1997) Targeted inhibition of interferon-gamma-dependent intercellular adhesion molecule-1 (ICAM-1) expression using dominant-negative Stat1. *J. Biol. Chem.*, **272**, 28582–28589.
50. Sandberg, E.M., Ma, X., VonDerLinden, D., Godeny, M.D. and Sayeski, P.P. (2004) Jak2 tyrosine kinase mediates angiotensin II-dependent inactivation of ERK2 via induction of mitogen-activated protein kinase phosphatase 1. *J. Biol. Chem.*, **279**, 1956–1967.
51. Wilkie, A.O., Patey, S.J., Kan, S.H., van den Ouweland, A.M. and Hamel, B.C. (2002) FGFs, their receptors, and human limb malformations: clinical and molecular correlations. *Am. J. Med. Genet.*, **112**, 266–278.
52. Aviezer, D., Golembo, M. and Yayon, A. (2003) Fibroblast growth factor receptor-3 as a therapeutic target for Achondroplasia—genetic short limbed dwarfism. *Curr. Drug Targets*, **4**, 353–365.
53. Dreyer, S.D., Zhou, G. and Lee, B. (1998) The long and the short of it: developmental genetics of the skeletal dysplasias. *Clin. Genet.*, **54**, 464–473.
54. Minina, E., Kreschel, C., Naski, M.C., Ornitz, D.M. and Vortkamp, A. (2002) Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. *Dev. Cell*, **3**, 439–449.
55. Coffin, J.D., Florkiewicz, R.Z., Neumann, J., Mort-Hopkins, T., Dorn, G.W., II, Lightfoot, P., German, R., Howles, P.N., Kier, A., O'Toole, B.A. *et al.* (1995) Abnormal bone growth and selective translational regulation in basic fibroblast growth factor (FGF-2) transgenic mice. *Mol. Biol. Cell*, **6**, 1861–1873.
56. Sahni, M., Raz, R., Coffin, J.D., Levy, D. and Basilico, C. (2001) STAT1 mediates the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF2. *Development*, **128**, 2119–2129.
57. Aikawa, T., Segre, G.V. and Lee, K. (2001) Fibroblast growth factor inhibits chondrocytic growth through induction of p21 and subsequent inactivation of cyclin E-Cdk2. *J. Biol. Chem.*, **276**, 29347–29352.
58. Tsukada, Y., Tanaka, T., Miyazawa, K. and Kitamura, N. (2004) Involvement of down-regulation of Cdk2 activity in hepatocyte growth factor-induced cell cycle arrest at G1 in the human Hepatocellular carcinoma cell line HepG2. *J. Biochem. (Tokyo)*, **136**, 701–709.
59. Venkatasubbarao, K., Choudary, A. and Freeman, J.W. (2005) Farnesyl transferase inhibitor (R115777)-induced inhibition of STAT3(Tyr705) phosphorylation in human pancreatic cancer cell lines require extracellular signal-regulated kinases. *Cancer Res.*, **65**, 2861–2871.
60. Wu, Y.Y. and Bradshaw, R.A. (1996) Induction of neurite outgrowth by interleukin-6 is accompanied by activation of Stat3 signaling pathway in a variant PC12 cell (E2) line. *J. Biol. Chem.*, **271**, 13023–13032.
61. Deng, C., Zhang, P., Harper, J.W., Elledge, S.J. and Leder, P. (1995) Mice lacking p21^{CIP1/WAF1} undergo normal development, but are defective in G1 checkpoint control. *Cell*, **82**, 675–684.
62. Ausubel, F.M., Brent, R.H., Myers Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.S. and Struhl, K. (1998) *Current Protocols in Molecular Biology*. Wiley, New York.
63. Feinberg, A.P. and Vogelstein, B. (1984) A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. *Anal. Biochem.*, **137**, 266–267.