Published in final edited form as:

Cell Signal. 2009 January ; 21(1): 151–160. doi:10.1016/j.cellsig.2008.10.006.

Fibroblast growth factor inhibits interferon γ -STAT1 and interleukin 6-STAT3 signaling in chondrocytes

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Abstract

Activation of fibroblast growth factor receptor 3 (FGFR3) leads to attenuation of cartilage growth. The members of the STAT family of transcription factors are believed to participate in FGFR3 signaling in cartilage, however the molecular mechanism of this action is poorly understood. Here, we demonstrate that a chronic FGF stimulus leads to accumulation of STAT1, 3, 5 and 6, evident in both in vitro chondrocyte model and murine limb explant cultures. Despite the accumulation, both endogenous and cytokine-induced activation of STAT1 and STAT3 is impaired by FGF, as demonstrated by imaging of active STAT nuclear translocation and analyses of STAT activatory phosphorylation and transcriptional activation. Further, we demonstrate that FGF induces expression of CIS, SOCS1 and SOCS3 inhibitors of gp130, a common receptor for the IL6-family of cytokines. Since cytokine-gp130 signaling represents an important positive regulator of cartilage, its inhibition may contribute to the growth-inhibitory effect of FGFR3 in cartilage.

Keywords

Fibroblast growth factor; fibroblast growth factor receptor 3; STAT1; STAT3; cartilage; chondrocyte; cytokine; inhibition

INTRODUCTION

Activating mutations in the tyrosine kinase FGFR3 result in several human skeletal dysplasias including thanatophoric dysplasia (TD) and achondroplasia (ACH), which are the most common genetic forms of lethal and non-lethal skeletal dysplasias, respectively [1]. Despite recent progress in characterization of the FGFR3-mediated regulation of cartilage, many aspects of this regulation remain unclear. A specific area of controversy is the precise role of the STAT family of transcription factors in FGFR3 signaling in cartilage. In the cartilage of

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ACH and TD-affected human fetuses as well as in murine models to ACH and TD, STATs accumulate and show nuclear localization, suggesting their activation [2-5]. In genetic studies, the loss of STAT1 partially rescued the FGFR3 effect on cartilage suggesting a role for STAT1 in FGFR3 signaling [6,7]. Since FGFR3 activation leads to chondrocyte growth arrest accompanied by induction of cell cycle inhibitors (CKI) [5,8], and activation of STAT1 leads to CKI induction [9], it has been hypothesized that FGFR3 activates STAT1 to induce CKIs that in turn inhibit chondrocyte proliferation [3]. In contrast, we demonstrate that while STAT1 can be directly phosphorylated by the FGFR3 in vitro, it does not participate nor is required for FGFR3-mediated inhibition of cell proliferation [10,11].

Given the complexity of growing cartilage, where several signaling systems influence chondrocyte proliferation and differentiation in an intricate spatiotemporal relationship, FGFR3-STAT signaling may affect chondrocyte behavior independent of regulation of proliferation. In addition, one limitation of in vitro experiments addressing mechanisms of FGFR3 signaling in chondrocytes is that they tend to elucidate the short-term effects of FGFR3 activation. This contrasts to the in vivo situation where chondrocytes are exposed to a chronic FGFR3 signal. This study was undertaken to unravel the effects of chronic FGFR3 activation on STAT signaling pathways in chondrocytes. Since in vitro cell lines used to experimentally evaluate STAT contribution to FGFR3 signaling do not model all the events regulated by FGFR3 in cartilage [11], we used a murine limb explant culture model to confirm the chondrocyte cell line findings.

MATERIALS AND METHODS

Cell and limb explant culture and Western immunoblotting (WB)

RCS chondrocytes were propagated in DMEM media (Gibco, Gaithersburg, MD), containing 10% FBS (Atlanta Biological, Nordcross, GA) and antibiotics. For limb explant cultures, forelimbs were isolated from E16.5 Balb/c murine embryos as described [12]. Briefly, forelimbs were stripped of skin and muscles, and cultured for up to four days in BGJb medium (Gibco) supplemented with antibiotic/antimycotic, and 0.1% BSA. They were grown in 6-well plates containing Cyclopore supports (Falcon Labware, Liverpool, Australia) under humidified conditions. In all cases, experiments were carried out on the left forelimb, with the right forelimb as an untreated control. For WB, cells were lysed in immunoprecipitation buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 0.5% NP-40, 1 mM EDTA, 25 mM NaF, 0.1 mM DTT, 1 µg/ml leupeptin, 10 µg/ml soybean trypsin inhibitor, 1 mM PMSF, 8 mM β-glycerolphosphate, 10 mM Na₃VO₄, 1 µg/ml aprotinin). Lysates were resolved by SDS-PAGE, transferred onto a PVDF membrane and visualized by luminescence (Amersham, Piscataway, NJ). The following antibodies were used: ACTIN and LIFR (Santa Cruz Biotechnology, Santa Cruz, CA); STAT1, STAT3, STAT5, STAT6, P-STAT1^{Y701}, P-STAT3^{Y705}, P-STAT5^{Y694}, JAK1, JAK2 and JAK3 (Cell Signaling, Beverly, MA); JAK1 (DB Biosciences, Franklin Lakes, NJ); SOCS1, SOCS3, CIS and P-STAT6^{Y641} (Abcam, Cambridge, UK); IFNγ R1, IFNγ R2 and IL11 Rα (R&D Systems, Minneapolis, MN). To quantify the WB signal, the integrated optical density (I.O.D) of a given band was determined using Scion Image software (Scion Corporation, Frederick, MA).

Signal transduction studies and STAT transcription factor assays

RCS chondrocytes cultivated in the presence of 10% FCS were treated for the desired time with FGF2 (40 ng/ml, unless specified otherwise), IL6 (20 ng/ml), IL2, IL4, IL5, IL11, IL13, GM-CSF, erythropoietin and growth hormone (all 40 ng/ml; R&D Systems), LIF (40 ng/ml; Chemicon, Temecula, CA) or IFNγ (40 ng/ml; Calbiochem, San Diego, CA). For STAT transcription factor assays, growing RCS cultures were treated as indicated and cell nuclei were isolated using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) as directed by the

manufacturer. The ELISA-based transcription factor assay (TransAM Stat Assay kit; Active Motif) was used to quantify the amount of active STAT1 and STAT3 in nuclear extracts. Briefly, the active STATs were purified from a nuclear lysate upon binding an immobilized oligonucleotide containing a 5'-TTCCCGGAA-3' motif, and detected by ELISA.

Vectors, cell transfection and confocal microscopy

The vectors containing STAT1 or STAT3 C-terminally fused to GFP or YFP were obtained from Drs H. Hauser, T. Miyazaki and G. Müller-Neven, respectively [13-15]. Before transfection, the RCS extracellular matrix was degraded by 0.3% bacterial collagenase (type II; Invitrogen, Carlsbad, CA). Cells were transfected with FuGENE6 (Roche Diagnostics, Penzberg, Germany) according to the manufacturer's protocol. For confocal microscopy, cells were transfected with STAT1-GFP or STAT3-GFP/YFP, grown for 24 hours, treated as indicated, fixed with 4% paraformaldehyde and mounted in media containing DAPI for nuclear staining (Vectashield; Vector Laboratories, Burlingame, CA). The confocal microscopy procedure is described in detail elsewhere [11]. To quantify the STAT-GFP/YFP nuclear translocation, cell cytoplasm and nucleus were manually selected and an integrated optical density (I.O.D) of selected area was determined using Scion Image software (Scion Corporation).

Real-time RT-PCR analysis

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and treated with DNase I. cDNA was synthesized from 2 µg of total RNA using Omniscript RT kit (Qiagen). Real-time RT-PCR conditions are described in detail elsewhere [8]. Table 1 lists the PCR primers used.

RESULTS

Chronic FGF stimulus leads to STAT accumulation in RCS chondrocytes

Rat chondrosarcoma chondrocytes (RCS) maintain a cartilage-like phenotype in vitro and express FGFR3 at high levels while they lack other FGFRs [6]. Since RCS chondrocytes represent the best studied cell model to FGFR3-related skeletal dysplasia to date [16-22], we used RCS cells in this study. To determine whether a chronic FGF stimulus leads to the STAT activation, we treated RCS cells with FGF2 for up to 6 days and assessed STAT1, 3, 5 and 6 activatory phosphorylation using western blotting (WB) with phosphorylation-specific antibodies. The specificities of used antibodies were determined previously [11]. Similar to short-term FGF2 treatment, no phosphorylation of STAT1(Y701), STAT5(Y694) or STAT6(Y641) was induced by FGF2 in contrast to STAT3(Y705) phosphorylation that appeared increased upon FGF2 treatment (Fig. 1A) [11]. However, the amount of total STAT3 also increased significantly in FGF2-treated cells similar to STAT1, 5 and 6 (Fig. 1A). The STAT accumulation correlated positively with increasing FGF2 dose (Fig. 1B).

Next, we addressed the mechanism underlying FGF2-mediated STAT accumulation. RCS chondrocytes were treated with FGF2 for up to 72 hours and cell lysates were analyzed for STAT protein and transcript expression by WB and real-time RT-PCR, respectively. Protein levels increased along with transcript levels (Fig. 2A), suggesting that increased transcription underlies the observed protein accumulation. When the amount of STAT transcript induction is compared with the corresponding protein, it appears that the transcriptional upregulation may not fully account for the protein accumulation (Fig. 2A). In another words, FGF2 might accumulate STATs at the protein level, independent of transcription. To test this prediction, we transfected RCS chondrocytes with vector expressing STAT3 N-terminally fused to yellow fluorescent protein (YFP) under the control of constitutively active pCMV promotor. Figure 2B demonstrates that STAT3-YFP is also accumulated following the chronic FGF2 treatment.

As ERK MAP kinase pathway represents the major pathway involved in FGFR3 signaling in cartilage [8,19], we tested whether it plays role in FGF2-mediated STAT accumulation in RCS chondrocytes as well. Inhibition of ERK pathway by chemical inhibitor of MEK kinase U0126 prevented the FGF2-mediated accumulation of all tested STATs (Fig. 2C).

Chronic FGF stimulus inhibits cytokine-mediated activation of STAT1 and STAT3

Given the significant accumulation of total STAT proteins in FGF2 treated RCS chondrocytes (Figs 1, 2), we asked whether this accumulation leads to enhanced cytokine signaling, which represent the major signaling systems that utilize STATs. We therefore treated RCS chondrocytes for 1 hour with interferon- γ (IFN γ), interleukin (IL)-6, IL11 and leukemia inhibitory factor (LIF) and determined the amounts of active nuclear STAT1 or STAT3 using an ELISA-based STAT transcription factor assay in the presence and absence of FGF2. Surprisingly, while STATs were activated following the cytokine treatment, this activation was significantly impaired by FGF2. In addition, basal STAT activity appeared suppressed by FGF2 as well (Fig. 3A) [11].

Figure 3A demonstrates that the cytokine-mediated activation of both STAT1 and STAT3 is inhibited by FGF2 despite the potent accumulation of total STATs in cells (Figs 1, 2). We therefore asked how FGF2 impairs the cytokine-STAT signaling pathways. IL6 added for 4 hours to culture media conditioned by FGF2-treated RCS chondrocytes for 48 hours induced normal activatory phosphorylation of STAT3(Y705), suggesting that FGF2 does not inhibit the IL6 signaling via some extracellularly released inhibitor (Fig. 3B). Since nuclear accumulation represents a hallmark of STAT activation, we expressed green fluorescent protein (GFP)-tagged STAT1 or STAT3 in RCS chondrocytes and examined the effect of chronic FGF2 stimulation (48h) on nuclear translocation of both STAT fusion proteins in cells treated with IFN γ or IL6 for up to 4 hours. The biological activity and expression of both STAT-GFP chimeras was confirmed before [11, 13, 14]. Figures 4B and 5B show both STAT-GFP chimeras are equally distributed between the nucleus and cytoplasm in cells growing in the presence of 10% FBS. Treatment with IFN γ or IL6 lead to progressive nuclear accumulation of both STATs. This accumulation was, however, significantly reduced by FGF2 for the majority of cytokine treatment times analyzed (Figs 4B, C and 5B, C). Since FGF2 induced significant accumulation of STATs, we tested whether the loss of cytokine-induced STAT1-GFP or STAT3-GFP nuclear translocation in cells treated with FGF2 can simply represent an artifact of higher STAT levels in cells. This would be unlikely in STAT1-GFP transfected cells that did not show overexpression of the transgene (Fig. 4A). In the case of STAT3-GFP or STAT3-YFP, however, RCS transfection lead to overexpression of the transgene, that was further elevated by FGF2 (Fig. 5A, D). Through manipulating the amount of transfected plasmid, we achieved STAT3-YFP expression in FGF2 naïve cells comparable to cells treated with FGF2 for 48h (Fig. 5D). When such cells were treated with IL6, complete nuclear translocation of STAT3-YFP was observed in contrast to cells pre-treated with FGF2 that showed full inhibition of STAT3-YFP nuclear translocation. These data demonstrate that the quantity of STAT-YFP expression does not affect the FGF2 inhibitory effect on its IL6-mediated nuclear translocation. We therefore conclude that FGF2 inhibition of cytokine-mediated activation of STATs (Fig. 3A) takes place at the level or upstream of STAT nuclear accumulation (Figs 4, 5).

Chronic FGF stimulus inhibits activatory tyrosine phosphorylation of STAT1 and STAT3

Cytokine binding to their cognate receptors induces receptor tyrosine phosphorylation via activation of their associated JAK kinases that in turn recruit and tyrosine phosphorylate STATs. This phosphorylation allows for STAT dimerization followed by nuclear translocation and DNA binding [23].

We next determined whether FGF2 interferes with STAT activatory tyrosine phosphorylation. Figure 6 shows that STAT3(Y705) phosphorylation induced by IL6 is almost completely inhibited by FGF2 pre-treatment (48h) despite FGF2-induced accumulation of total STAT3. Similar to IL6, the IFN γ -mediated phosphorylation of STAT1(Y701) was reduced by FGF2 although this inhibition appeared later and was less pronounced when compared to IL6.

As STAT5 and STAT6 were also upregulated by a chronic FGF2 stimulus (Figs 1, 2), we tested whether FGF2 inhibits cytokine-mediated activation of STAT5 and STAT6 similar to STAT1 and STAT3. First we treated RCS chondrocytes with a panel of cytokines including IL2, IL4, IL5, IL6, IL11, IL13, GM-CSF, LIF, IFN γ , erythropoietin and growth hormone and used WB to detect the activatory Y694 or Y641 phosphorylation of STAT5 or STAT6, respectively. We did not detect STAT5(Y694) phosphorylation in RCS chondrocytes regardless of the cytokine treatment in contrast to STAT6, which was phosphorylated at Y641 after IL13 treatment (data not shown). We then evaluated the inhibitory effect of FGF2 on this phosphorylation using the same experimental design as shown on Fig. 6A. We found no inhibitory effect of FGF2 treatment on IL13-mediated STAT6(Y641) phosphorylation of RCS cells (Fig. 6C).

Chronic FGF stimulus induces inhibitors of cytokine signaling

Having demonstrated that FGF2 inhibits IFN γ and IL6 signaling at the level of STAT phosphorylation, we tested whether this inhibition might be a consequence of FGF2 effects upon mediators of the cytokine signaling pathways. Real-time RT-PCR was used to assess the expression of specific components of these pathways in the presence and absence of FGF2. The studied genes included the receptor genes (*gp130*, *Ifngr1*, *Ifngr2*, *Il6ra*, *Il11ra* and *Iifr*), their associated kinases (*Jak1*, *Jak2*) and their inhibitors (*Socs1*, *Socs3*, *Cis*, *Shp1*, *Shp2* and *Ptp1b*). Figure 7A demonstrates that FGF2 induced expression of *Socs1*, *Socs3* and *Cis* as well as down-regulation of the *Il6ra*. Expression of the other selected genes was unaffected (data not shown). We next used WB to test whether SOCS1, SOCS3 and CIS were induced by FGF2 also at the protein level. Fig 7B shows FGF2-mediated upregulation of both SOCS1 and SOCS3 whereas no CIS protein was detected in RCS chondrocytes with the used antibody. In addition, we found an inhibitory effect of FGF2 on LIFR protein expression (Fig. 7B). JAK1, JAK2 and IFNGR1 protein expression was not affected by FGF2 and we did not detect IL11RA or IFNGR2 in RCS chondrocytes by the used antibodies (data not shown).

Chronic FGF stimulus causes STAT accumulation and inhibits IL6-mediated STAT3 activatory phosphorylation in murine limb explant cultures

In order to validate the accumulation of STATs and impairment of cytokine signaling observed in the RCS chondrocytes, we determined the FGF2 effect on cytokine-STAT signaling in murine limb explant cultures [12]. Forelimbs isolated from E16.5 Balb/c murine embryos were first treated with 50 ng/ml of FGF2 for up to 4 days and analyzed for STAT accumulation by WB. Figure 8A shows that FGF2 accumulated each STAT tested (STAT1, 3, 5 and 6), albeit to a lesser extent than in RCS chondrocytes. Next, we pre-treated the limb explants with FGF2 for 48 hours followed by 30 minutes of IL6 treatment. Figure 8B shows that FGF2 increases the basal level of phosphorylated STAT3(Y705) in limb explant cultures, similar to RCS chondrocytes (Fig. 1). However, IL6-mediated STAT3(Y705) phosphorylation was significantly inhibited by FGF2, again confirming the RCS data (Figs 6A, 8B).

DISCUSSION

The data presented here describe an interaction of FGF and cytokine signaling in chondrocytes that results in the upregulation of STAT1, 3, 5 and 6, and in the case of STAT3, an increased tyrosine phosphorylation following FGF2 treatment (Figs 1, 2, 8). Although STAT upregulation might be expected to enhance canonical STAT signaling, we found the opposite,

i.e. inhibition of cytokine mediated activation of STAT1 and STAT3 in the presence of FGF2 (Figs 3-9). This inhibition appears to be due to the FGF2-mediated induction of CIS, SOCS1 and SOCS3 inhibitors of cytokine receptors as well as suppression of IL6RA and LIFR expression (Figs 7, 9).

FGF2-mediated upregulation of STATs in chondrocytes

In RCS chondrocytes as well as in mouse limb explant cultures, increased STAT1, 3, 5 and 6 protein was detected (Figs 1, 2, 8) after at least 12 hour of FGF2 treatment. These protein increases were at least partially accompanied by increased transcript levels, suggesting that FGF2 induces a transcriptional upregulation of STAT gene expression. Why would chondrocytes upregulate STAT transcription with FGF2 treatment? Using different experimental approaches, we demonstrate that FGF2 inhibits “basal” STAT1 and STAT3 activity in RCS chondrocytes, which is likely a result of serum-borne or autocrine cytokine stimulation (Figs 3A, 6B) [11]. As the time-course of FGF2-mediated inhibition of basal STAT1/3 activity correlates precisely with STAT1/3 accumulation (Fig. 2A) [11], we hypothesize that RCS chondrocytes upregulate STAT1/3 expression in an attempt to compensate for inhibition of basal STAT1/3 activity by FGF2. In addition to transcriptional accumulation, our experiments suggest that a portion of FGF2-mediated STAT1/3 accumulation occurs at the protein level, and that ERK MAP kinase is a candidate for this effect (Fig. 2B, C). ERK is known to phosphorylate STATs at a conserved P(M)SP motif located between residues 720 and 730 and this phosphorylation takes place in FGF2-treated RCS chondrocytes [24,11]. Although we do not know the significance of ERK-mediated phosphorylation for STAT protein accumulation, we speculate that it may increase STAT protein stability, perhaps by allowing STATs to associate with other cytoplasmic protein(s). Experiments are now ongoing to test this hypothesis.

What might be the consequence of FGF-mediated upregulation of STATs? In the growth plate cartilage obtained from patients suffering from ACH or TD, the accumulation and preferential nuclear localization of STAT1 as well as p21^{Cip1} was observed, which correlated positively with disease severity, (i.e. the level of activation of FGFR3), thus leading to conclusion that FGFR3 upregulates STAT1-p21^{Cip1} to inhibit chondrocyte proliferation [3]. In RCS chondrocytes, we observe similar STAT1 and p21^{Cip1} accumulation that also correlates positively with the level of FGFR3 activation (Fig. 1B) [8]. However, FGF2-mediated STAT1 and p21^{Cip1} accumulation may not be of a functional relationship in RCS chondrocytes since STAT1 does not contribute to the FGF-mediated growth arrest that is caused by p38 and ERK MAP kinases [8,11,19].

FGF2-mediated inhibition of cytokine signaling in chondrocytes

Using several different experimental approaches, i.e. imaging of STAT1/3 nuclear accumulation, a STAT1/3 transcription factor assay and WB with phosphorylation-specific antibodies, we have shown that chronic FGF2 stimulation inhibits IL6-, IL11-, LIF- and IFN γ -STAT1/3 signaling (Fig. 9). Similar to others [22], we found that FGF2 induces expression of three members of the CIS-SOCS family: CIS, SOCS1 and SOCS3 (Fig. 7). CIS-SOCS proteins function as E3 ubiquitin ligases that associate with a cytokine-receptor complex to trigger its ubiquitin-mediated degradation. In addition, SOCS1 and SOCS3 can directly inhibit JAKs via their kinase inhibitory region that acts as a pseudosubstrate [25]. Since SOCS1 prefers IFN γ receptor, whereas SOCS3 associates preferentially with gp130 [26,27], FGF2-mediated induction of SOCS1 and SOCS3 may explain its inhibitory effect on both IFN γ and IL6-mediated phosphorylation of STAT1(Y701) and STAT3(Y705), respectively (Figs 6A, 9).

Despite the fact that both STAT5 and STAT6 were also upregulated by the FGF2 treatment of RCS chondrocytes, it is unclear to what extent the FGF2 signaling interferes with cytokine-mediated activation of STAT5/6. We could not test the influence of FGF2 on STAT5 activation due to our inability to find a STAT5-activating cytokine. We found that STAT6 is activated by IL13 in RCS chondrocytes, but this activation was not inhibited by FGF2 (Fig. 6C). These data suggest that the inhibitory effect of FGF2 on cytokine-STAT signaling might be cytokine-specific rather than general and/or limited to STAT1 and STAT3.

Altogether, we find that chronic FGF2 signaling inhibits IFN γ -STAT1 and IL6/IL11/LIF-STAT3 signaling in chondrocytes. Although the precise role of IFN γ in cartilage is not yet defined, the IL6-family cytokines represent important regulators of cartilage. This is evidenced by the existence of a human dwarfism condition, Stüve-Wiedemann syndrome (MIM#601559), which arises from null mutations in LIF receptor [28], and also by the phenotype of knock-in mice expressing gp130 unable to signal via STAT1/3 (*gp130^{ΔSTAT/ΔSTAT}*) [29]. Such animals show markedly shortened limbs due to reduced chondrocyte proliferation and premature growth plate closure, demonstrating the essential positive role of gp130-STAT1/3 signaling in cartilage growth. Since FGFR3 inhibits both proliferation and cytokine-gp130 signaling in chondrocytes (Fig. 9), we hypothesize that FGFR3 partly inhibits cartilage growth via inhibition of gp130 signaling.

CONCLUSIONS

In this article, we address the effects of FGF signaling on cytokine-STAT signaling in a chondrocyte cell environment as a model for FGFR3-related skeletal dysplasias. We conclude that: A) Chronic FGF stimulation leads to STAT accumulation, demonstrated in both in vitro chondrocyte model and murine limb explant cultures. B) Despite the accumulation, both endogenous and cytokine-induced activation of STAT1 and STAT3 is impaired by FGF, likely via the induction of several inhibitors of cytokine-STAT signaling such as CIS, SOCS1 and SOCS3. Since cytokine-STAT signaling represents an important positive regulator of cartilage, its inhibition may contribute to the growth-inhibitory effect of FGFR3 in cartilage.

Acknowledgements

We are grateful to Drs H. Hauser, T. Miyazaki and G. Müller-Neven for STAT vectors, to Dr. M. Schibler for confocal microscopy, to Dr. R. Pogue for preparation of murine limb explant cultures and to P. Mekikian for excellent technical assistance. This work was supported by National Institutes of Health (5P01-HD22657), Yang Sheng Tang USA company, Multiple Myeloma Research Foundation, the Ministry of Education, Youth and Sports of the Czech Republic (MSM0021622430), a Cedars-Sinai Medical Genetics Institute fellowship (PK), and a Winnick Family Research Scholars award (WRW).

References

1. Passos-Bueno MR, Wilcox WR, Jabs EW, Sertie AL, Alonso LG, Kitoh H. Hum Mutat 1999;14:115–125. [PubMed: 10425034]
2. Legeai-Mallet L, Benoist-Lasselin C, Delezoide A, Munnich A, Bonaventure J. J Biol Chem 1998;273:13007–13014. [PubMed: 9582336]
3. Legeai-Mallet L, Benoist-Lasselin C, Munnich A, Bonaventure J. Bone 2004;34:26–36. [PubMed: 14751560]
4. Chen L, Adar R, Yang X, Monson E, Li C, Hauschka PV, Yaron A, Deng C. J Clin Invest 1999;104:1517–1525. [PubMed: 10587515]
5. Li C, Chen L, Iwata T, Kitagawa M, Fu X, Deng C. Hum Mol Genet 1999;8:35–41. [PubMed: 9887329]
6. Sahni M, Ambrosetti D, Mansukhani A, Gertner R, Levy D, Basilico C. Genes Dev 1999;13:1361–1366. [PubMed: 10364154]

7. Sahni M, Raz R, Coffin JD, Levy D, Basilico C. *Development* 2001;128:2119–2129. [PubMed: 11493533]
8. Krejci P, Bryja V, Pachernik J, Hampl A, Pogue R, Mekikian P, Wilcox WR. *Exp Cell Res* 2004;297:152–164. [PubMed: 15194433]
9. Chin YE, Kitagawa M, Su WS, You ZH, Iwamoto Y, Fu XY. *Science* 1996;272:719–722. [PubMed: 8614832]
10. Nowroozi N, Raffioni S, Wang T, Apostol BL, Bradshaw RA, Michels Thompson L. *Hum Mol Genet* 2005;14:1529–1538. [PubMed: 15843401]
11. Krejci P, Salazar L, Goodridge HS, Kashiwada TA, Schibler M, Jelinkova P, Michels Thompson L, Wilcox WR. *J Cell Sci* 2008;121:272–281. [PubMed: 18198189]
12. Minina E, Wenzel HM, Kreschel C, Karp S, Gaffield W, McMahon AP, Vortkamp A. *Development* 2001;128:4523–4534. [PubMed: 11714677]
13. Köstner M, Hauser H. *Eur J Biochem* 1999;260:137–144. [PubMed: 10091593]
14. Watanabe K, Saito K, Kinjo M, Matsuda T, Tamura M, Kon S, Miyazaki T, Uede T. *Biochem Biophys Res Commun* 2004;324:1264–1273. [PubMed: 15504351]
15. Herrmann A, Sommer U, Pranada AL, Giese B, Haan S, Becker W, Heinrich PC, Müller-Neven G. *J Cell Sci* 2003;116:339–349. [PubMed: 14657276]
16. Aikawa T, Segre GV, Lee K. *J Biol Chem* 2001;276:29347–29352. [PubMed: 11384971]
17. Rozenblatt-Rosen O, Mosonogo-Ornan E, Sadot E, Madar-Shapiro L, Sheinin Y, Ginsberg D, Yayon A. *J Cell Sci* 2002;115:553–562. [PubMed: 11861762]
18. Dailey L, Laplantine E, Priore R, Basilico C. *J Cell Biol* 2003;161:1053–1066. [PubMed: 12821644]
19. Raucci A, Laplantine E, Mansukhani A, Basilico C. *J Biol Chem* 2004;279:1747–1756. [PubMed: 14593093]
20. Krejci P, Masri B, Fontaine V, Mekikian PB, Weis M, Prats H, Wilcox WR. *J Cell Sci* 2005;118:5089–5100. [PubMed: 16234329]
21. Priore R, Dailey L, Basilico C. *J Cell Physiol* 2006;207:800–808. [PubMed: 16523491]
22. Ben-Zvi T, Yayon A, Gertler A, Monsonego-Ornan E. *J Cell Sci* 2005;119:380–387. [PubMed: 16410555]
23. Aaronson DS, Horvath CM. *Science* 2002;269:1653–1655. [PubMed: 12040185]
24. Decker T, Kovarik P. *Oncogene* 2000;19:2628–2637. [PubMed: 10851062]
25. Yoshimura A, Naka T, Kubo M. *Nat Rev Immunol* 2007;7:454–464. [PubMed: 17525754]
26. Yamamoto K, Yamaguchi M, Miyasaka N, Miura O. *Biochem Biophys Res Commun* 2003;310:1188–1193. [PubMed: 14559241]
27. Qing Y, Costa-Pereira AP, Watling D, Stark GR. *J Biol Chem* 2005;280:1849–1853. [PubMed: 15522878]
28. Dagoneau N, Scheffer D, Huber C, Al-Gazali LI, Di Rocco M, Godard A, Martinovic J, Raas-Rothschild A, Sigaudy S, Unger S, et al. *Am J Hum Genet* 2004;74:298–305. [PubMed: 14740318]
29. Sims NA, Jenkins BJ, Quinn JM, Nakamura A, Glatt M, Gillespie MT, Ernst M, Martin TJ. *J Clin Invest* 2004;113:379–389. [PubMed: 14755335]

FIGURE 1. FGF2 causes STAT accumulation in chondrocytes

(A) RCS chondrocytes were treated with FGF2 for the indicated times and cell lysates were analyzed for STAT1, STAT3, STAT5 and STAT6 by WB. Note that the accumulation of total STAT protein in FGF2-treated cells correlates with the appearance of the phosphorylated form in STAT3 (P-Y705-STAT3) but not in the other STATs analyzed. The membrane used for STAT5 detection was reprobed for ACTIN immunoreactivity as a loading control. (B) RCS chondrocytes were treated with FGF2 for 72 hours and cell lysates were analyzed for STAT levels by WB with the signal quantitated by densitometry (charts). STAT3, STAT5 and STAT6 accumulation correlate positively with the FGF2 dose. The membrane used for STAT5 detection was reprobed for ACTIN immunoreactivity as a loading control. The results are representative for three experiments.

FIGURE 2. FGF2 causes STAT accumulation at both the transcript and protein level

(A) RCS chondrocytes were treated with FGF2 for the indicated times, and duplicate cell lysates were analyzed for STAT expression at both the protein and transcript levels using WB (upper blot) and real-time RT-PCR (lower chart), respectively. The real-time RT-PCR data represent the fold difference over untreated cells. The data represent technical duplicates with the indicated range. The results are representative for three experiments. Note the time correlation of both STAT protein and transcript accumulation. The membrane used for STAT5 detection was reprobbed for ACTIN immunoreactivity as a loading control. (B) RCS chondrocytes were transfected with STAT3-YFP vector, treated with FGF2 for the indicated times and analyzed for STAT3 quantities by WB. Note that FGF2 accumulated also the vector-borne STAT3-YFP, whose expression is driven by a pCMV promoter. ACTIN serves as a loading control. (C) RCS chondrocytes were pretreated with ERK pathway inhibitor (U0126; 20 μ M) four hours prior to FGF2 treatment for 48 hours. Note that the FGF2-mediated STAT accumulation was prevented by U0126. ACTIN serves as a loading control.

FIGURE 3. FGF2 inhibits cytokine-mediated transcriptional activation of STAT1 and STAT3
 (A) RCS chondrocytes were pretreated with FGF2 for 72 hours and treated with IL6, IL11, LIF or IFN γ for 1 hour. Cell nuclei were analyzed for active STAT1 (upper graph) or STAT3 (lower graph) by STAT transcription factor assay as described in the Material and Methods. STAT activation was quantified by spectrophotometry. FGF2 has an inhibitory effect on both basal and cytokine-mediated activation of STATs. The data represent an average from two samples with the indicated range. The dashed line indicates endogenous STAT nuclear activity in untreated cells. The results are representative for three experiments. (B) RCS chondrocytes were treated for indicated times with media conditioned by confluent control or FGF2-treated (48 hours) RCS culture, and analyzed for STAT3 activatory phosphorylation (P-Y705-STAT3) by WB. IL6 was incubated with the conditioned media for 4 hours. Total STAT3 quantities serve as a loading control. Note that IL6-mediated activation of STAT3 is not impaired by the media conditioned by FGF2-treated cells suggesting that FGF2 does not inhibit STAT3 via an extracellularly released inhibitor.

FIGURE 4. FGF2 inhibits cytokine-mediated nuclear accumulation of STAT1-GFP

(A) RCS chondrocytes were transfected with STAT1-GFP vector and the expression of the transgene was confirmed by WB 48 hours later (lane 2). (B) RCS chondrocytes were transfected with STAT1-GFP, treated with FGF2 for 48 hours prior to IFN γ treatment for the indicated times, fixed and analyzed for STAT1-GFP sub-cellular distribution by confocal microscopy. DAPI staining indicates the cell nucleus. Note the FGF2-mediated inhibition of IFN γ -mediated nuclear accumulation of STAT1-GFP. Bar = 10 μ m. (C) The subcellular STAT1-GFP distribution shown in (B) was quantified as described in Materials and Methods, with at least 15 cells analyzed for each time point. Statistically significant differences are highlighted (Student's *t*-test, **P*<0.01). The results are representative for three experiments.

FIGURE 5. FGF2 inhibits cytokine-mediated nuclear accumulation of STAT3-GFP or STAT3-YFP

(A) RCS chondrocytes were transfected with STAT3-GFP vector and the expression of transgene was confirmed by WB 48 hours later. Note the appearance of STAT3-GFP as well as its degradation products in transfected cells (lane 2) in comparison to untransfected control (lane 1). (B) RCS chondrocytes were transfected with STAT3-GFP vector, treated with FGF2 for 48 hours prior to IL6 treatment for 1 hour, fixed and analyzed for STAT3-GFP sub-cellular distribution by confocal microscopy. DAPI staining indicates the cell nucleus. Note the FGF2-mediated inhibition of IL6-mediated nuclear accumulation of STAT3-GFP. Bar = 10 μ m. (C) The subcellular STAT3-GFP distribution shown in (B) was quantified as described in Material and Methods, with at least 15 cells analyzed for each time point. Statistically significant differences are highlighted (Student's *t*-test, **P*<0.01). The results are representative for three experiments. (D) RCS chondrocytes were transfected with the indicated amounts of STAT3-

YFP vector and the expression of the transgene was confirmed by WB 48 hours later (C - untransfected control). Note that transfection no. 2 leads to STAT3-YFP overexpression comparable to transfection no. 3 followed by FGF2 treatment (48 hours; lane 4). (E) RCS chondrocytes were transfected with STAT3-YFP vector as indicated in (D), treated with IL6 for 1 hour, fixed and analyzed for STAT3-YFP sub-cellular distribution by confocal microscopy. DAPI staining indicates cell nucleus. Note the IL6-mediated nuclear accumulation of entire amount of overexpressed STAT3-YFP (transfection no. 2). The FGF2 inhibition of IL6-mediated nuclear accumulation of STAT3-YFP was comparable to STAT3-GFP (C). (F) The subcellular STAT3-YFP distribution shown in (E) was quantified as described in Materials and Methods, with at least 15 cells analyzed for each time point. Statistically significant differences are highlighted (Student's *t*-test, * $P < 0.01$).

FIGURE 6. FGF2 inhibits IFN γ - or IL6-mediated activation of STAT1/3 but not IL13-mediated activation of STAT6

(A) RCS chondrocytes were treated with FGF2 for 48 hours prior to cytokine treatment and analyzed for activatory STAT1(Y701) or STAT3(Y705) phosphorylation by WB. Controls represent cells not treated with the cytokine at the beginning (t=0) or the end of the experiment (t=4h). The blots were reprobed for total STAT1 or STAT3 as well as for ACTIN that serves as a loading control. Note that the FGF2 inhibitory effect on cytokine-mediated STAT activation is more profound in IL6-treated cells when compared to IFN γ at the initial stimulation times (<30 minutes). The results are representative for three experiments. (B) RCS cultures prepared parallel to those shown above were analyzed for active nuclear STAT1 by a STAT transcription factor assay as described in the Materials and Methods to control for the FGF2 inhibitory effect. STAT activation was quantified by spectrophotometry. The data represent an average from two samples with the indicated range. (C) RCS chondrocytes were treated similarly to (A) and analyzed for activatory STAT6(Y641) phosphorylation by WB. Controls represent cells not treated with IL13 at the beginning (t=0) or the end of the experiment (t=4h). The blots were reprobed for total STAT6 as well as for ACTIN that serves as a loading control. Note the lack of FGF2 inhibitory effect on IL13-mediated STAT6 activation.

FIGURE 7. FGF2 upregulates inhibitors of cytokine signaling

(A) RCS chondrocytes were treated with FGF2 for the indicated times and the amounts of *Socs1*, *Socs3*, *Cis* and *Il6ra* transcripts were determined by real-time RT-PCR. Transcript quantities are relative to untreated cells. The data represent an average from technical duplicates with the indicated range. The results are representative for five experiments. (B) RCS chondrocytes were treated with FGF2 for 48 hours and the amounts of LIFR, SOCS1 and SOCS3 were determined by WB. ACTIN serves as loading control. Note the inhibitory effect of FGF2 on the LIFR expression as well as the FGF2-mediated induction of SOCS1 and SOCS3. The data show three control and three FGF2-treated RCS cultures.

FIGURE 8. FGF2 accumulates STATs and inhibits IL6-mediated STAT3 activatory phosphorylation in murine limb explant cultures

(A) Limb explant cultures were initiated and maintained as described in the Materials and Methods. The cultures were treated with FGF2 for indicated times and analyzed for STATs by WB. The WB signal was quantitated by densitometry. The membrane used for STAT5 detection was reprobbed for ACTIN immunoreactivity as a loading control. (B) Limb explant cultures were treated with FGF2 for 48 hours prior to IL6 treatment (30 minutes) and analyzed for activatory STAT3(Y705) phosphorylation by WB. The blots were reprobbed for total STAT3 as well as for ACTIN, which serves as a loading control. The WB signal was quantitated by densitometry. The results are representative for two (A) or three (B) experiments.

FIGURE 9. FGF2-mediated inhibition of cytokine-STAT signaling in chondrocytes

In their signaling pathways, IFN γ , IL6, IL11 and LIF activate STATs via binding to their cognate receptor coupled to gp130 or IFNGR1 and associated STAT kinases. FGF2 is proposed to interfere with STAT activation through induction of three cytokine receptor inhibitors, CIS, SOCS1 and SOCS3. In addition, IL6 and LIF signaling is further impaired by FGF2-mediated downregulation of the IL6 and LIF cognate receptors, IL6RA and LIFR.

Table 1

PCR primers used for real-time RT-PCR analyses

Gene	Forward (5' to 3')	Reverse (5' to 3')	Product (bp)	Annealing (°C)
<i>Cis</i>	TCCCCAGATGTGGTCAGCCTTG	GACTAGCCGACACAGGTGTTGTAA	231	65
<i>Gapdh</i>	ACCACAGTCCATGCCATCAC	TCCACCACCCTGTGCTGTA	452	65
<i>gp130</i>	CCATACTTTCAGGCTATCCTCCAG	TGGGTTTCACTTTATCCACGGG	309	65
<i>Ifngr1</i>	TGTGAAACATTACAGGAGTGGGG	AGAGGAGCAACAAGCAGCATCC	243	65
<i>Ifngr2</i>	CCTGTTCCCTCAGATACCAAAGCC	TCAGTGTCTCCGTGTAAGCAGAG	248	65
<i>Il6ra</i>	GGAAGAACCCCTTGTAATATGC	CCAACTGACTTTGAGCCAACGSAG	349	63
<i>Il11ra</i>	TTGGAGGAGTTGATAACAGATGCC	GATGCTAACACAGCCACTTGCTC	290	63
<i>Jak1</i>	CCTATTTCCCGAGATTACCCAC	TTGGACCGTGACAGCCATTCTG	231	63
<i>Jak2</i>	CCAAATGGAGAGTATGTTGCCG	ACAAAGTCGTCAAGGAGAGGAGC	275	63
<i>Lifr</i>	TCTTTATGGGGAGACTGTCGCC	TTCAAACCTGTGGAACACTGCC	286	63
<i>Ptp1b</i>	CAAACGCACATTGGAGCCTC	TCTTCCTCAGTGGGGACAGATG	231	65
<i>Shp1</i>	TTGATATGCTCATGGAGAGCGTC	TTGATATGCTCATGGAGAGCGTC	228	65
<i>Shp2</i>	AGCCGTTACCCACATCAAGATTC	GCTGTCATTGCTCTCCCCTTTG	343	65
<i>Socs1</i>	TTCGAGCTGCTGGAGCACTACGTG	TGGAAGGGAAGGAACTCAGG	188	65
<i>Socs3</i>	CGGATTCTACTGGAGTGCCGTAAC	CAACTTGAGTACACAGTCAAAGCGG	238	65
<i>Stat1</i>	GGCTTGGTGATTGACCTTGAGAC	TCTTGGTGACTGACGAAAACCTGC	208	65
<i>Stat3</i>	TGATGCCTCCTTGATTGTCCTG	CGTTCTTGGGGTTATTGGTCAAGC	191	65
<i>Stat5</i>	TGTCCCTGAAACGAATCAAGAGG	TAGCCGTCGCATTGTTGTCCTG	186	65
<i>Stat6</i>	AACGGCTCTATGTTGACTTTCCAC	GATGCTCTCCAAGGTGCTGATG	215	65