

Induction of chondrocyte growth arrest by FGF: transcriptional and cytoskeletal alterations

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Summary

The effect of fibroblast growth factor (FGF) on mature chondrocytes, the cells responsible for axial skeletal development, is growth attenuation rather than stimulation. This singular response has been linked to signaling via FGF receptor 3 (FGFR3), partly because mutations causing chronic FGFR3 activation lead to various human disorders of bone growth. In order to study how FGF inhibits growth, we analyzed its effect on a rat chondrocyte-derived cell line. We show that the FGF-induced growth arrest occurs at the G1 phase, accompanied by profound changes in gene expression and cytoskeletal organization. Within minutes of binding, FGF induces tyrosine kinase activity in the focal substrate adhesions where it colocalizes with vinculin. Upon FGF stimulation, FGFR3 is selectively removed from the focal adhesions, which is followed by their disassembly and disruption of the organized cytoskeleton. Multiple genes are induced following FGF stimulation in chondrocytes, which has been shown by DNA array screening and

confirmed for some by immunoblotting. These genes include regulators of cell differentiation and proliferation such as c-jun, JunD, cyclin-D1, NFκB1 and of plasma-membrane microdomain morphology, such as ezrin. The transcription factor Id1 is downregulated, consistent with the cells' exit from the mitotic cycle. Moreover, following FGF stimulation, levels of FGFR3 mRNA and protein decline, as does downstream signaling through the MAPK pathway. The importance of this FGFR3-mediated on-off control is illustrated in transgenic mice expressing mutant, hyperactive FGFR3, where abnormally high levels of NFκB are expressed throughout their bone growth-plates. A working model is presented of the signaling network involved in regulating FGF-induced chondrocyte differentiation and receptor downregulation.

Key words: Chondrocytes, FGF signaling, FGF receptor 3, Focal adhesions, G1 arrest

Introduction

Fibroblast growth factor receptor 3 (FGFR3) is one of four distinct members of the membrane-spanning tyrosine kinase family that serve as high affinity receptors (FGFRs) for at least 20 different fibroblast growth factors (FGFs). Binding of the FGF ligand, in concert with heparan sulfate, induces FGFR dimerization and transphosphorylation, followed by activation of downstream signal-transduction pathways (reviewed in Givol and Yayon, 1992; Burke et al., 1998; Klint and Claesson-Welsh, 1999; Ornitz, 2000). Signaling via the FGF-FGFR system plays a major role in regulating various cellular processes, including proliferation, differentiation and survival. In vitro, the effect of FGF on most cell types, such as endothelial cells (Gerwins et al., 1998), fibroblasts (Basilico and Moscatelli, 1992) or de-differentiated chondrocytes (Kato and Iwamoto, 1990; Hill et al., 1991; Wroblewski and Edwall-Arvidsson, 1995), is stimulation of proliferation and inhibition of terminal differentiation. However, the effect of FGF on growth-plate chondrocytes, the cells responsible for bone growth and skeletal development, and Rat Chondrosarcoma (RCS) cells, which retain mature chondrocyte markers and express high levels of FGFR3, is potent inhibition of proliferation (Sahni et al., 1999), which may ultimately result in apoptosis (Legeai-Mallet et al., 1998).

The expression of FGFRs is tightly regulated during embryonal development and tissue regeneration (Basilico and Moscatelli, 1992; Yamaguchi et al., 1995; Goldfarb, 1996; Martin, 1998; Szebenyi and Fallon, 1999; Xu et al., 1999). FGFR3 is particularly highly expressed during embryonic development in the pre-cartilaginous mesenchyme (Peters et al., 1992; Peters et al., 1993) and later on in the maturation zone of the epiphyseal growth-plates, where it is involved in long bone development (Naski et al., 1998). The discovery that specific activating mutations in FGFR3 underlie a variety of human skeletal disorders, such as Achondroplasia, the most common form of human genetic dwarfism, has linked FGFR3 signaling and skeletal development (reviewed in Webster and Donoghue, 1997; Burke et al., 1998; Naski and Ornitz, 1998). Moreover, FGFR3-null mice exhibit bone overgrowth accompanied by expansion of proliferating and hypertrophic chondrocytes within the growth-plate (Colvin et al., 1996; Deng et al., 1996). Transgenic mice harboring FGFR-activating mutations (Naski et al., 1998; Chen et al., 1999; Li et al., 1999; Wang et al., 1999; Segev et al., 2000) or overexpressing FGF2 (Coffin et al., 1995) or FGF9 (Garofalo et al., 1999) display a dwarf phenotype similar to the human disorders where attenuated proliferation and

differentiation of chondrocytes result in retarded bone growth (Naski et al., 1998; Chen et al., 1999; Li et al., 1999). Overall, these studies indicate that FGFR3 acts as a potent regulator of chondrocyte differentiation and as a negative regulator of bone growth. However, the downstream events by which FGFR3 influences the proliferation or terminal differentiation of chondrocytes remains poorly understood.

Although the role of cell cycle regulating proteins in maintaining the balance between proliferation and differentiation is well studied, there is limited data on the expression pattern of cell-cycle-regulating genes during chondrocyte maturation (LuValle and Beier, 2000). The expression of the *p21^{Waf1/Cip1}* gene, a cyclin-dependent kinase inhibitor, was found to be upregulated during chondrocyte differentiation in vitro (Beier et al., 1999) and in vivo (Stewart et al., 1997) and to be controlled by FGFs along with activation of the transcription factor STAT1 in RCS cells (Sahni et al., 1999). Stat-1-null mice, however, have not been reported to have bone defects (Durbin et al., 1996; Meraz et al., 1996). In this study, we have utilized a chondrosarcoma model cell system to further study the FGF-mediated control mechanisms. We show that FGF signaling and growth arrest induces alterations in the subcellular localization of FGFR3, and several candidate genes that may be involved in the regulation of the cell cycle and cytoskeletal organization.

Materials and Methods

Cell culture

Rat Chondrosarcoma (RCS) cells were grown in Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FCS). Treated cells were supplemented with FGF 9 (20 ng/ml) and heparin (1 µg/ml).

Western blot analysis

Cells were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5), 150 mM NaCl, 1 mM EDTA, 10 % glycerol, 1% NP40 and 'Complete' (Boehringer Mannheim) protein inhibitor mix according to the manufacturers instructions). Equal amounts of cell lysates, as determined by a Bradford reaction, were loaded, resolved by SDS-PAGE and followed by western blot analysis. Proteins were visualized by using an enhanced chemiluminescence kit (ECL Amersham). Anti-FGFR3 antibodies, anti-NF-κB p65 antibodies, anti-c-Jun antibodies, anti-JunD antibodies. Anti-FRS2 antibodies were a generous gift from Yaron Hadari, and anti-Id antibodies were from Santa Cruz biotechnology. Anti-Ezrin antibodies were from Transduction Laboratories. Anti-pMAPK antibodies and preimmune rabbit serum were from Sigma. Secondary antibodies used were anti-rabbit or -mouse immunoglobulins linked to horseradish peroxidase (Amersham Life Science).

Cell cycle analysis

Cells were trypsinized, centrifuged, resuspended in 300 µl PBS, fixed in 5 ml methanol and stored at -20°C for at least 24 hours. On the day of analysis, cells were collected by centrifugation and washed with 1 ml PBS. Cell pellets were then resuspended in PBS (between 0.5-1ml) containing 100 µg/ml RNase and 12.5 µg/ml propidium iodide and incubated in the dark for 20 minutes. Filtered samples were then analyzed for cell cycle content by using FACsort (Becton-Dickinson).

Screening of Atlas cDNA expression array

Total RNA was extracted from untreated RCS cells or RCS cells that were incubated with 20 ng/ml FGF9 and 1 µg/ml heparin by using a Tri Reagent kit (Molecular Research Center). After DNase treatment, P³²-radiolabeled cDNA was prepared from 9 µg of total RNA and hybridized to the membranes (Clontech, 7738-1) according to the manufacturer's instructions.

Immunohistochemistry

Isolated bones from mouse hind limbs were fixed in 4% paraformaldehyde (pH 7.4), decalcified in EDTA, dehydrated in an ethanol gradient and embedded in paraffin. 5-µm thick sections were cut, dewaxed in xylene, hydrated through graded ethanol to water and then rinsed in PBS. Endogenous peroxidase activity was quenched by incubation in 3% hydrogen peroxide for 10 minutes. Immunostaining was performed using the Histostain-plus broad-spectrum peroxidase kit (Zymed Laboratories). Sections were blocked in normal serum for 10 minutes and incubated with anti NFκB p65 polyclonal antibody diluted 1:200 in PBS overnight at 4°C. Sequentially, the sections were incubated with biotinylated secondary antibodies, followed by avidin horseradish peroxidase conjugate and diaminobenzidine substrate as a chromogen. Finally, the sections were counterstained with methyl green, dehydrated in graded ethanols, cleared in xylene and mounted. Negative controls for immunostaining were performed by substitution of the primary antibody with PBS or preimmune serum.

Immunofluorescence

Cells were cultured on glass coverslips for 48 hours, permeabilized with 0.5% TritonX-100 and fixed with 3% paraformaldehyde in phosphate-buffered saline. Fixed cells were incubated with the relevant primary antibodies to FGFR3, pTYR (Upstate) and vinculin (Transduction Laboratories) and detected using Cy3 goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories) and Alexa 488 goat anti-mouse IgG (Molecular probes) as secondary antibodies. Actin was detected by direct phalloidin (Sigma) staining. Image acquisition was performed using an Axiphot (Zeiss) microscope equipped with an AttoArc (Zeiss) camera using a 100× objective (Zeiss) or with the DeltaVision system (Applied Precision, Issaquah, WA, USA) equipped with a Zeiss Axiovert 100 microscope (Oberkochen, Germany) and photometrics 300 series scientific-grade cooled CCD camera (Tucson, AZ, USA) reading 12 bit images using a 100×1.3 NA plan-Neoflaur objective (Zeiss, Oberkochen, Germany).

RNA preparation and northern blot analysis

Total RNA was extracted from cells using the EZ-RNA kit (Biological Industries) according to the manufacturer's instructions. 15 µg of total RNA were loaded on a 1% agarose MOPS/formaldehyde gel and transferred to a nylon membrane (Sartorius). The blot was hybridized overnight to ³²PdCTP-labeled FGFR3 cDNA probe (nucleotides 60-667) in NorthernMax buffer (Ambion), washed and exposed to a Biomax film (Kodak).

Results

FGF9 activates FGFR3, downregulates its expression and induces G1 arrest in RCS cells

RCS cells exhibit several of the properties of mature chondrocytes, as they express chondrocyte-specific markers, including collagen type II, alkaline phosphatase and high levels of FGFR3. To investigate the signal-transducing pathways activated by FGFR3 in chondrocytes, cells were incubated with FGF9 and heparin for various time periods and monitored by

western blotting for activation of the downstream signaling effectors ERK 1,2 and FRS2/SNT and for FGFR3 expression. ERK kinase phosphorylation was undetectable in untreated cells and became evident 10 minutes after stimulation with FGF9. FGFR3 activation was followed using an antibody against SNT/FRS2 proteins and their tyrosine-phosphorylated forms. A 77 kDa band compatible in size with that expected for phospho-FRS2 beta was identified with an antibody that recognizes both the alpha and the beta forms (Kouhara et al., 1997). This band became detectable within 10 minutes, and its intensity was further increased three hours after FGF9 addition. This band was also confirmed to be tyrosine-phosphorylated in separate experiments (not shown) that involved immunoprecipitation with anti-FRS2 followed by immunoblotting with anti-phosphotyrosine. In unstimulated RCS cells, the anti-FRS2 antibody bound predominantly to a protein of 60-65 kDa. Since the *Mr* of this protein is below that reported for activated FRS2 alpha and it was not detectable by anti-phosphotyrosine antibodies (not shown), it is most likely the non-activated form of FRS2 beta (Xu et al., 1998).

We found, in agreement with Sahni et al. (Sahni et al., 1999), that FGF9, a more specific ligand for FGFR3 (Hecht et al., 1995), not only induces activation of the receptor but also results in rapid downregulation of its expression. A profound decrease in the level of the FGFR3 protein was already detected 4 hours after addition of the ligand and fell below detection levels after 8 hours. ERK phosphorylation decreased concomitantly, with complete loss of this downstream signal after 8 hours (Fig. 1A). Northern blot analysis demonstrated that the level of the transcript encoding for FGFR3 decreased with similar kinetics (Fig. 1B), possibly indicating the induction of a transcriptional inhibitory loop mechanism.

Long-term exposure of RCS cells to FGF9 resulted in a dramatic inhibition of their proliferation (Fig. 1C); a similar effect had previously been observed for FGF1 (Sahni et al., 1999). The density of the cell cultures incubated with FGF9 for various periods (8, 16, 24 and 72 hours) was essentially unchanged from the initial seeding density, as estimated by microscopic examination, although some dead cells were observed following incubations of more than 16 hours (data not shown). This qualitative observation indicated that FGF9 caused cell growth arrest. Following removal of FGF9 and continuation of growth for a further 72 hours, the numbers of cells in the cultures were found to be 7.2×10^5 , 6.8×10^5 , 3.5×10^5 , 2.9×10^5 and 1.1×10^5 for FGF exposures of 0, 8, 16, 24 and 72 hours, respectively. Since the original number of cells seeded was 0.6×10^5 in each case, we conclude that a significant proportion of the cells exposed to FGF9 for 8-24 hours was capable of restarting growth after removal of FGF9. However, cell death and apoptosis can not be ruled out for the cells that failed to regain proliferative capacity.

In order to analyze the growth inhibition in greater detail, RCS cells were incubated with or without FGF9 for 16 hours and subjected to FACS analysis. Cells incubated with FGF9 either alone or together with heparin exhibited a significant increase in the percentage of cells in the G1 phase (86% compared with 62% in untreated cells; Fig. 2A) and a concomitant decrease in the percentage of cells in S phase (5% compared with 22% in the untreated cells; Fig. 2A), strongly suggesting that FGF9 induces growth arrest at the G1 phase of the cell cycle. Addition of heparin alone had no effect on the

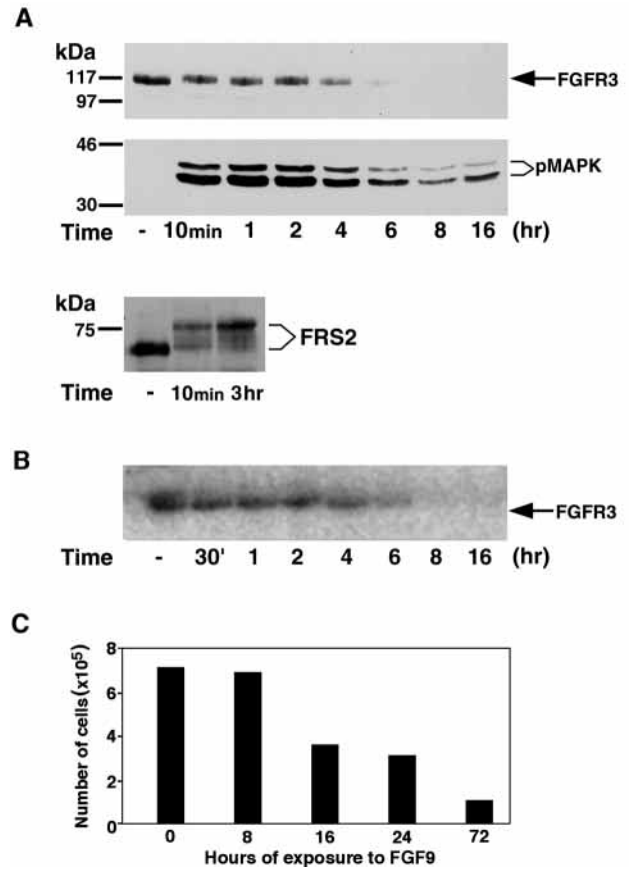


Fig. 1. Effects of FGF9 on RCS cell proliferation, FGFR3 activation and gene expression. RCS cells were harvested at the indicated time points following stimulation with 20 ng/ml of human recombinant FGF9 and 1 μ g/ml of heparin. (A) Equal amounts of cell lysates were analyzed by SDS-PAGE and western blotting with anti-FGFR3 antibodies (upper panel), anti-pMAPK antibodies (middle panel) and anti-FRS2 antibodies (lower panel). (B) Cells were harvested, and RNA was prepared and subjected to northern blot analysis using human FGFR3 cDNA as a probe. (C) RCS cells (60,000 cells/well) were incubated with FGF9 and heparin for 0 (untreated control), 8, 16, 24 and 72 hours as indicated. Then, FGF9 was washed out and the cells were cultured further under normal conditions for 72 hours, at which point the cell number was determined. The initial cell number seeded in each system was 0.6×10^5 . The respective cell numbers obtained at the end of the 72-hour incubation were 7.2×10^5 , 6.8×10^5 , 3.5×10^5 , 2.9×10^5 and 1.1×10^5 . Values are averages of triplicates, with standard deviation <10% in all cases.

cell cycle (Fig. 2A). In a detailed time course analysis, a transient accumulation of the surviving cells at the G2 phase of the cell cycle was noted after 8 hours of treatment (28% compared with 13% in the untreated cells) (Fig. 2B), preceding the G1 growth arrest detected after 10 hours of incubation with FGF9.

FGF signaling modulates multiple genes in RCS cells

In an attempt to identify the genes that are modulated by FGF and that might be involved in FGF-induced growth arrest, we utilized DNA array technology (Fig. 3A). Total RNA extracted from RCS cells before (0 hours) and after incubation with

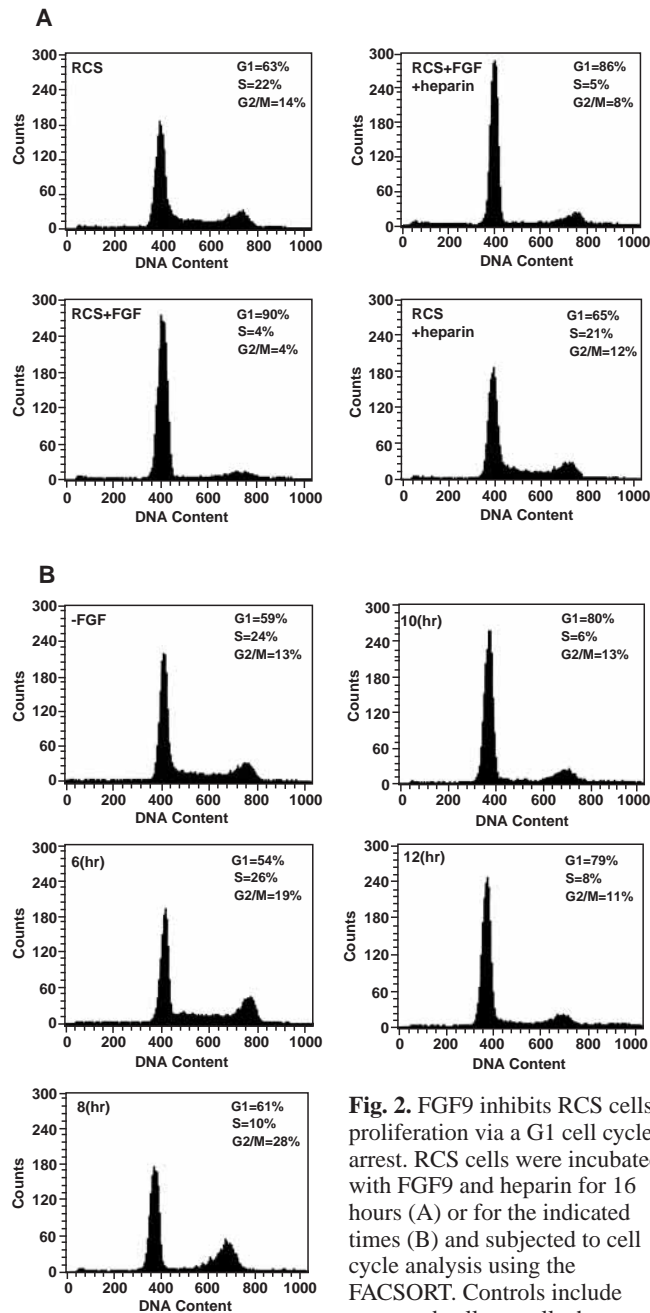


Fig. 2. FGF9 inhibits RCS cells proliferation via a G1 cell cycle arrest. RCS cells were incubated with FGF9 and heparin for 16 hours (A) or for the indicated times (B) and subjected to cell cycle analysis using the FACSORT. Controls include untreated cells or cells that were

incubated in the presence of heparin. The percentage of cells accumulated at the G1, S and G2/M stages of the cell cycle is indicated.

FGF9 and heparin for 3 hours was used for screening an Atlas membrane containing 588 known rat cDNAs (7738-1, Clontech). This array is composed mainly of genes reported to play key roles in processes such as signal transduction, apoptosis, tumor suppression and oncogenesis. The screen identified 11 distinct genes whose expression level changed more than two-fold upon FGF9 stimulation. Five of these were known FGF target genes, including c-Jun (Pertovaara et al., 1993; Cao et al., 1998), the urokinase receptor (Mignatti et al., 1991), the LDL receptor (Hsu et al., 1994), cyclin D1 (Rao et al., 1995) and p21 (Sahni et al., 1999). Among the genes that were significantly upregulated but had not been previously

described as direct FGF targets were JunD, FRA 2, NF- κ B1 (p50/p105), STAT3 and Ezrin. The expression of Id1 was markedly decreased (Fig. 3A). Computerized analysis of the data using AtlasImage 1.01 software (Clontech) demonstrated that the induction of c-Jun, JunD, FRA 2, cyclin D1, NF- κ B1(p50/p105), STAT3 and Ezrin was 2.45-, 15.13-, 5.63-, 2.27-, 3.55-, 3.05- and 3.98-fold higher, respectively. There was a threefold reduction in the expression of the *Id1* gene.

We confirmed the above results by determining the levels of the protein products of some of the genes in immunoblots (Fig. 3B). In agreement with the gene array results, the levels of c-Jun, JunD and p21 proteins increased at 2 hours after FGF addition and peaked at 4 hours. The increase in the levels of Ezrin was detectable only 6 hours after stimulation, and it remained high for at least 24 hours. The level of Id1 protein fell to below detection limit within 2 hours of treatment (Fig. 3B).

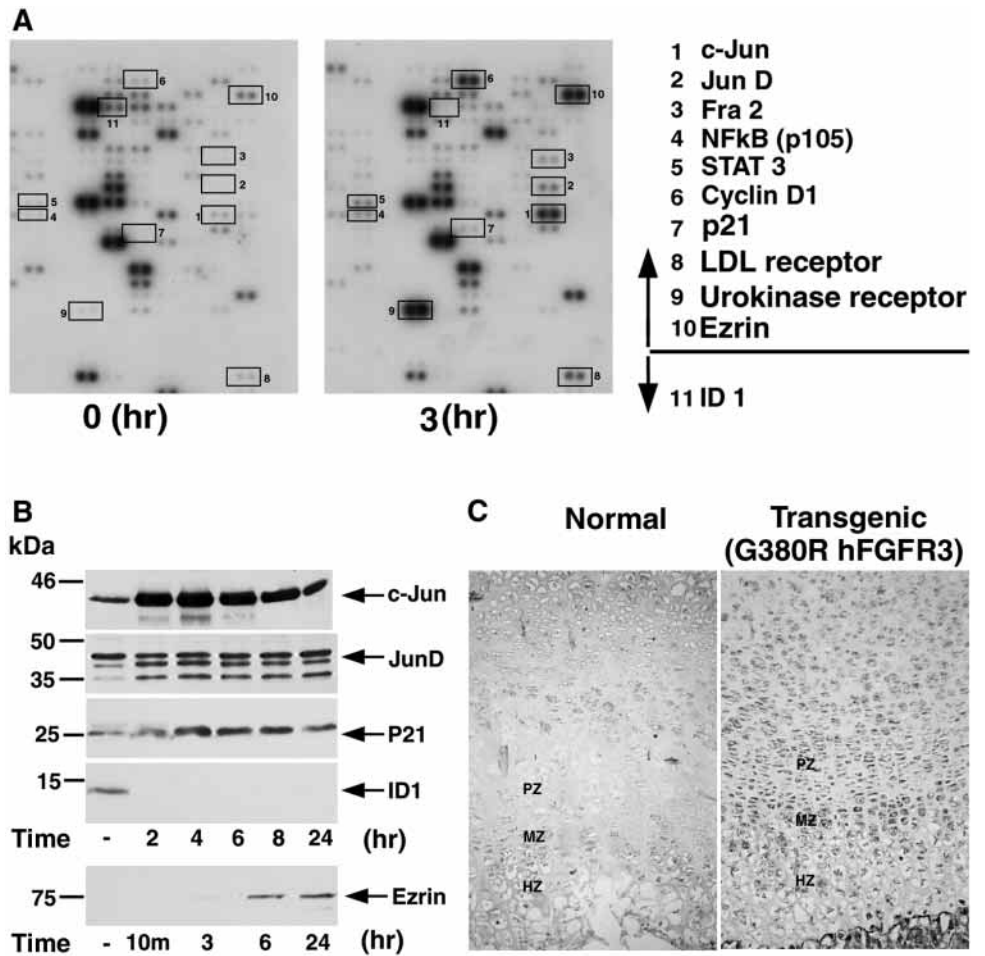
In a search for a possible link between the expression of some of the induced genes and FGFR3 activation *in vivo*, we have analyzed epiphyseal growth-plates from normal and from transgenic mice carrying the Achondroplasia *G380R hFGFR3* gene. Immunostaining of paraffin-embedded sections with anti Rel A (p65) antibodies (Rel A together with NF- κ B1(p50/p105) forms the active NF- κ B dimer) revealed a significantly denser distribution of Rel A in the growth-plates of the transgenic mice than in their normal littermates (Fig. 3C). Furthermore, the protein was predominantly expressed, like FGFR3, in the maturation/upper hypertrophic zones of the growth-plate. Immunostaining of growth-plate sections with antibodies against NF- κ B1(p50/p105) and c-Jun also showed similar qualitative differences (data not shown), although these were significantly less pronounced than for Rel A (p65). These results indicate that the constitutively active, mutant FGFR3 induces *in vivo* gene expression, which parallels that observed in FGF9-stimulated RCS cells.

FGF activates FGFR3 localized to focal adhesions and disrupts the cytoskeletal organization

It is well known that proliferation and cell cycle progression are tightly associated with cell shape and the organization of the cytoskeleton (Assoian and Zhu, 1997). This, together with the fact that FGF signaling induces the expression of Ezrin, a prominent cytoskeletal protein, led us to examine more carefully the morphological changes induced in these cells by FGF. In general, the majority of RCS cells in culture have a polygonal shape, which is typical of mature chondrocytes. A small percentage of RCS cells in the culture exhibit round morphology, which might represent a different differentiation stage. Incubation of RCS cells with FGF9 dramatically changed their morphology, with complete rounding of the cells apparent 6 hours after stimulation, whereas untreated cells or cells treated with heparin alone retained their polygonal shape (Fig. 4A).

The fact that the cells seem to at least partially detach from the plate prompted us to examine the effect of FGF9 on the organization of their focal adhesions, the major anchor sites of cells to their substrate. RCS cells were therefore subjected to double immunofluorescence staining with an anti-FGFR3 polyclonal antibody and an anti-vinculin monoclonal antibody (Fig. 4B). Unexpectedly, we found that a significant portion of

Fig. 3. Modulation of gene expression by FGF9. (A) Differential hybridization to Atlas rat gene array. Total RNA prepared from either RCS cells incubated with FGF9 and heparin for three hours or untreated cells was used to generate two radiolabeled cDNA probes. The probes were hybridized to the Clontech Atlas membranes (7738-1) as described in Materials and Methods. Each cDNA is present on the filter in two adjacent spots. The frames indicate several genes regulated by FGFR3. The previously unreported, upregulated genes were (fold-induction is given in brackets) c-Jun ($\times 2.5$), Jun D ($\times 15.1$), Fra 2 ($\times 5.6$), cyclin D1 ($\times 2.3$), NF- κ B1 (p50/p105) ($\times 3.6$), STAT3 ($\times 3.1$) and Ezrin ($\times 4.0$), whereas ID1 was downregulated three-fold. (B) Kinetics of protein expression following FGF9-induced growth arrest. RCS cells were exposed to FGF9 and heparin for the indicated time intervals, lysed and analyzed by SDS-PAGE and western blotting with anti c-Jun, Jun-D, p21, Id1 and Ezrin antibodies.



(C) Immunohistochemical analysis of epiphyseal growth plates from normal and transgenic G380R mutant h-FGFR3 expressing mice. Immunostaining for Rel A (NF- κ B p65) was performed on sections of proximal tibia growth plates from normal and transgenic littermates harboring the G380R mutated FGFR3. The different zones of the growth plate (PZ-proliferation zone, MZ-maturation zone, HZ-hypertrophic zone) are noted.

the FGFR3 protein was localized in arrowhead-shaped structures typical of focal adhesions and colocalized with vinculin. Notably, not all focal adhesions contain the receptor. Next, we examined whether activation of FGF signaling enhances phosphotyrosine (pTyr) activity at the focal adhesions by immunofluorescent staining of FGF9-stimulated cells with anti-phosphotyrosine antibodies (Fig. 5A). It is well documented that pTyr activity is abundant in the focal adhesions of unstimulated cells (reviewed in Vuori et al., 1998; Cary and Guan, 1999), as can clearly be seen in untreated RCS cells. Detailed time-course analysis showed that pTyr activity in the focal adhesions increases 10 minutes after FGF9 addition, which correlated with activation of FGFR3 and its downstream targets in the focal adhesions. This activity decreased after 1 hour and almost completely disappeared after 6 hours (Fig. 5A).

Most dramatic, however, was the disruption of the focal adhesions and its correlation with the kinetics of FGFR3- and vinculin-associated focal adhesions following FGF stimulation (Fig. 5B). Although 10 minutes after stimulation FGFR3 was still associated with the focal adhesions, an hour later it was almost undetectable in these sites. This was in marked contrast with vinculin which was retained in these adhesion sites, and its distribution was apparently unchanged up to 6 hours after stimulation as the cells became more rounded and the focal

adhesions disintegrated (Fig. 5B). Staining with phalloidin, which labels actin filaments, demonstrated a similar pattern whereby exposure to FGF9 for several hours induced a major breakdown of the organized actin network in these cells, as well as in ruffling and lamellipodia extensions, which were observed already one hour after FGF stimulation (Fig. 5C).

Discussion

Arrest of proliferation in response to FGF is characteristic of mature growth-plate chondrocytes and constitutes a trigger for their differentiation program that culminates in bone elongation. A central transducer of the FGF signal in chondrocytes appears to be FGFR3, as inborn mutations in this receptor lead to severe impairment of skeletal development. Two syndromes of human dwarfism, Achondroplasia and Thanatophoric dysplasia, are thought to be mainly caused by gain-of-function mutations in FGFR3, resulting in hyperactivation (Webster and Donoghue, 1997; Naski and Ornitz, 1998) and stabilization (Monsonego-Ornan et al., 2000) of the receptor. The implication of this and other observations are that, if following its initial FGF-mediated activation FGFR3 is not downregulated and its signaling is not shut-off, the chondrocyte differentiation program is disrupted. Presumably, therefore, growth arrest by FGF is an essential

mechanism for synchronizing the cells at a specific stage so they can undergo simultaneous maturation and differentiation. This study represents an attempt to obtain an overview of the molecular and cellular events that accompany the multifactorial responses of chondrocytes to FGF.

Cell cycle regulation and alterations in transcriptional pattern induced by FGF

We have chosen a rat chondrosarcoma cell line (RCS) as an *in vitro* model system because it expresses a high level of FGFR3 protein as well as other chondrocyte-specific markers (Sahni et al., 1999). The validity of this model is confirmed by the inhibition of RCS cell proliferation in response to FGF9, a considerably specific ligand for FGFR3 (Hecht et al., 1995). We show that the mitotic arrest occurs at the G1 phase of the cell cycle (Fig. 2). Interestingly, a careful analysis of the cell cycle data suggests that just before the G1 arrest, the cells transiently accumulate at the G2 phase (Fig. 2B), a mechanism shown previously to be directly associated with cell differentiation (Aloni-Grinstein et al., 1995; Schwartz and Rotter, 1998). Downregulation of FGFR3 in response to FGF was also confirmed for RCS cells by the dramatic reduction in the levels of FGFR3 mRNA and protein following the addition of FGF9 (Fig. 1). Furthermore, a consequence of the failure to turn off FGFR3 signaling, such as occurs with the constitutively activated G380R mutant of FGFR3 (Monsonogo-Ornan et al., 2000), is shown in Fig. 3C.

Stimulation by FGF, as is the case with all growth factors, has a profound effect on the cellular gene transcription profile. In the exploratory screen carried out in the present work, FGF was found to activate multiple genes and to repress one. Since FGF arrests chondrocyte proliferation and acts as a differentiation trigger, it is not unexpected that seven of the ten induced proteins (c-jun, Jun D, Fra2, NF κ B, STAT 3, Cyclin D1 and p21) as well as the repressed Id1 are involved in cell cycle regulation. c-Jun, Jun D and Fra1 are members of the AP-1 family of transcription factors (reviewed in Karin et al., 1997; Leppa and Bohmann, 1999), which are upregulated by FGF in RCS cells. Several studies have indicated that c-Jun and JunD inhibit the differentiation of chondrocytes *in vitro* (Kameda et al., 1997). In addition, Jun D plays a major role in

osteoblast maturation (McCabe et al., 1996) and has been implicated as a negative regulator of cell proliferation in several other cell types (Wang et al., 1996). The Fra2-related transcription factor, Fra1, is associated with enhanced osteoblast differentiation, resulting in increased bone formation (Jochum et al., 2000). Although the observed elevation in cyclin D1 mRNA levels does not typically correlate with a G1 arrest, FGF has been found to suppress MCF-7 human breast cancer cell proliferation concomitantly with an increase in cyclin D1 (Wang et al., 1997). Also, cyclin D1 is directly activated by the transcription factor ATF-2, which inhibits chondrocyte proliferation in mice (Beier et al., 1999). Therefore, this array of genes may participate in regulating cell cycle progression in order to establish the differentiation phenotype of mature chondrocytes.

Induction of the NF- κ B transcription factor and the cyclin inhibitor p21^{Waf1/Cip1} is usually associated with stress or injury.

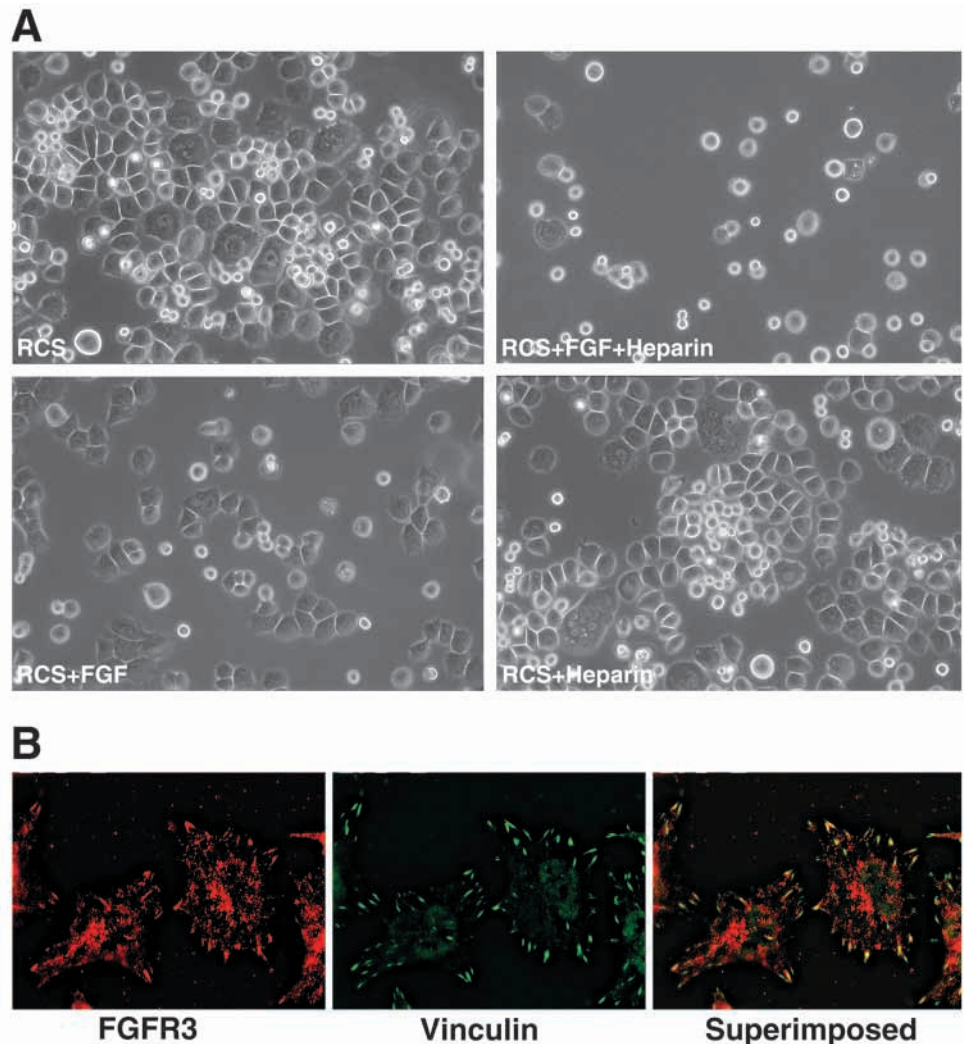


Fig. 4. FGF9 induces changes in RCS cell morphology and the subcellular localization of FGFR3. (A) RCS cells were stimulated with FGF9, heparin or FGF9 with heparin for 48 hours and visualized by phase microscopy. (B) Cells were subjected to double-labeled immunofluorescent staining with anti-FGFR3 and anti-vinculin antibodies followed by reaction with a secondary anti-rabbit antibody conjugated to Cy3 and anti-mouse conjugated to Alexa 488 antibodies, respectively. Coimmunofluorescence of green and red signals identifies the sites where the two proteins colocalize. A hypass filter was used in order to emphasize staining of the focal adhesions.

In chondrocytes, as other cells, NFκB has been shown to attenuate Fas-mediated apoptosis (Beg et al., 1995). Therefore, in the epiphyseal growth plate it may play a permissive role, by allowing the cells to reach maturity while inhibiting entry into the apoptosis pathway. The p21^{Waf1/Cip1} protein blocks the cell cycle and therefore may have a role in the FGF-induced G1 arrest. In this respect, its rise may serve a similar function to the fall in the levels of the Id1 protein, whose

downregulation has been associated with decreased mitotic activity in chondrocytes (Asp et al., 1998). The levels of NFκB in chondrocytes of transgenic mice expressing this mutation are dramatically elevated throughout the epiphyseal growth plates compared to those of control littermates. The phenotypic consequence of the G380R mutation is restrained chondrocyte proliferation and maturation, leading to inhibition of bone growth and dwarfism (Segev et al., 2000; Deng, 1996). It

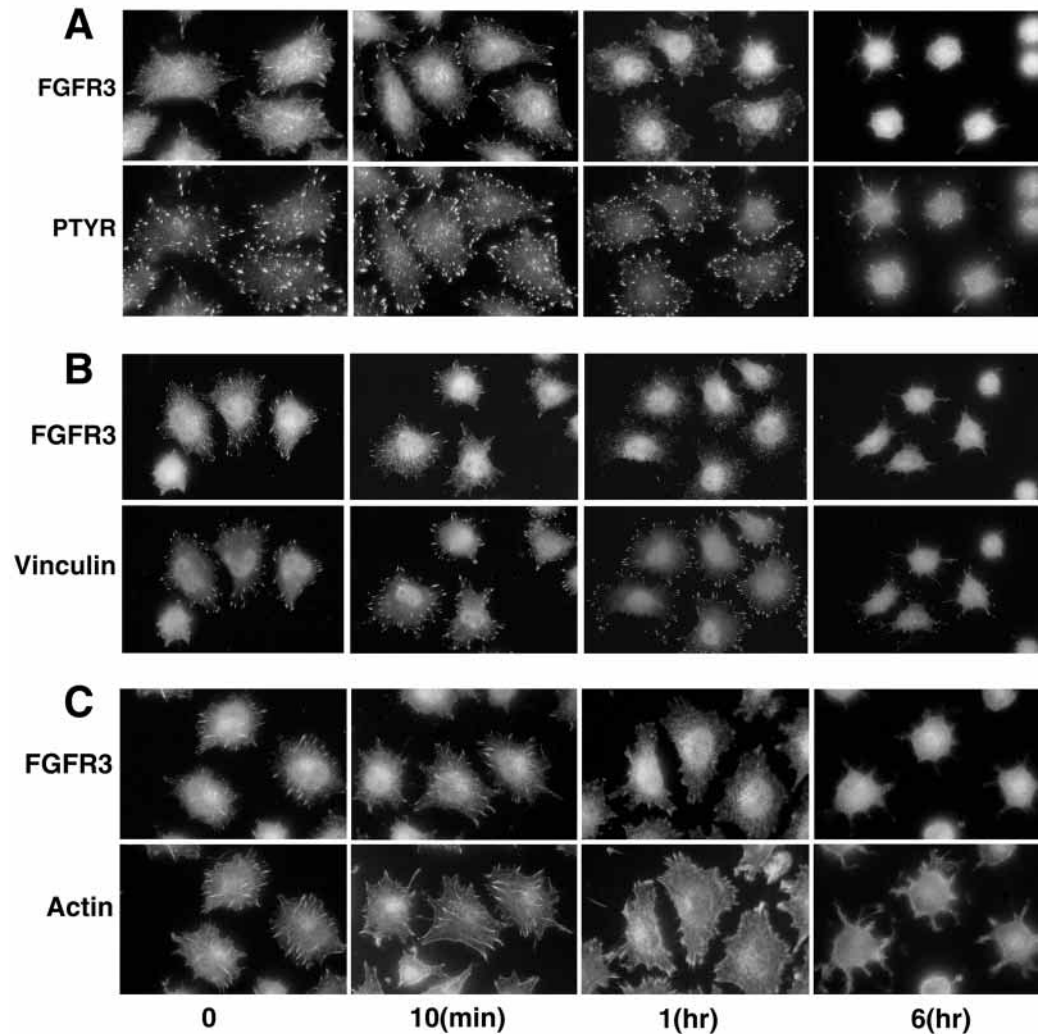
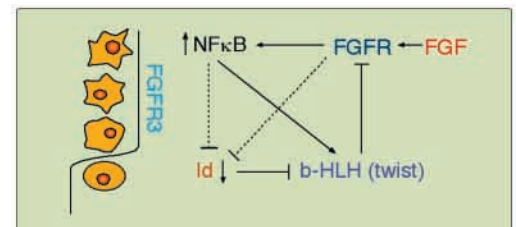


Fig. 5. Effects of FGF9 on RCS cell morphology, cytoskeletal organization and FGFR3 localization. RCS cells were incubated with FGF9 and heparin for 10 minutes, 1 hour or 6 hours, fixed and double stained with antibodies to FGFR3 and anti-pTYR antibodies (A) or anti-vinculin antibodies (B) or phalloidin (C).

Fig. 6. A schematic model suggesting how NFκB, Id1 and possibly Twist may interact to control FGFR gene expression. In this hypothetical scheme, it is proposed that the feedback regulation of FGFR expression in RCS cells involves principally NFκB and Id, acting via an unknown intermediary factor, which may be a b-HLH protein such as (Twist). Activation of FGFR induces upregulation of NF-κB subunits NF-κB1 (p50/p105) and RelA(p65) (Ghosh et al., 1998) and downregulation of Id1, a general inhibitor of terminal differentiation, which was shown to inhibit Twist by direct interaction with the protein. Several studies have demonstrated that activated NF-κB can upregulate Twist protein either directly or through inhibition of BMP4 signaling, which can directly regulate the expression of Id1. Since the involvement of Twist in this cell system is unknown and hypothetical, it has been placed in parentheses. FGFR may also downregulate the expression of Id1 via a NF-κB-independent signaling pathway. Both downregulation and signaling shut-off of FGFR3 are tightly regulated during chondrocyte maturation and terminal differentiation. 'FGFR3' refers to the total signal-transduction activity mediated via FGFR3, determined by the sum of activated FGFR3 molecules in the cell. The left side of the figure denotes alterations in chondrocyte morphology and their correlation with FGFR3 activity. Initially, FGFR3 signalling level is high and is associated with a transition in chondrocyte morphology from a polygonal to a rounded shape. In the last stage of the process (bottom cell), FGFR3 is downregulated and its signalling activity ceases as the cells attain a fully rounded shape.



remains to be determined to what extent the excessive expression of the pleiotropic activator NF κ B is responsible for this phenotype.

Members of the Id family of transcription factors function as general inhibitors of terminal differentiation by directly inactivating basic helix-loop-helix proteins (bHLH), thus controlling cell growth (Massari and Murre, 2000). Id1 and Id3 were already suggested to be involved in the control of proliferation and differentiation of cartilage (Asp et al., 1998). Most recently, Id1 and Twist, a b-HLH transcription factor family member, and a known downstream effector of NF- κ B (Tickle, 1998), were suggested to regulate the expression of FGFR2 during osteoblast maturation. In this system, FGF stimulation was proposed to induce Twist, which in turn is thought to inhibit FGFR2 and is counteracted by the action of Id (Rice et al., 2000). Moreover, loss-of-function mutations in the *Twist* gene in humans result in the Saethre-Chotzen syndrome, which is characterized by craniofacial and limb anomalies (Dixon et al., 1997). Although most patients with this syndrome have Twist-related mutations, some patients with an overlapping phenotype have a mutation either in *FGFR3* or *FGFR2* (Paznekas et al., 1998). *Drosophila* Twist is also thought to inhibit DFR1, the fly FGF receptor homologue (Shishido et al., 1993). In addition, NF- κ B inhibits signaling by BMP4 (Tickle, 1998), a factor that directly regulates the expression of Id1 (Hollnagel et al., 1999). BMP4, on the other hand, is downregulated by FGFR3 in the growth-plates of transgenic mice harboring the Achondroplasia mutant FGFR3 (Naski et al., 1998), which is consistent with their excessive NF κ B levels (Fig. 3C). We hypothesize that the FGF-induced upregulation of NF- κ B and reduction in Id1 in RCS cells mark their entry into the differentiation pathway. This signaling network is schematized in the partial working model presented in Fig. 6, where NF- κ B together with Id1 and a b-HLH protein, such as Twist, interact to turn off FGFR3.

FGFR3 localization, the cytoskeleton and growth control

Chondrocytes produce a thick cartilage matrix containing collagen and sulfated proteoglycans (Cancedda et al., 1995), which can serve as a substrate for focal adhesions. Although the functional importance of the clustering of FGFR3 in focal adhesions is unclear, it may be analogous to the aggregation of FGFR1 in focal adhesions isolated from endothelial cells using immobilized beads coated with a synthetic RGD tripeptide or with fibronectin (Plopper et al., 1995). FGFR2 is expressed, although not in this location in RCS cells (not shown), suggesting selectivity for FGFR3. The FGFR3-FGF complexes are most likely active in the focal adhesions, as evidenced by the increased tyrosine-phosphorylation in their immediate vicinity and the subsequent alterations in their structure. Among the most prominent focal-adhesion-resident signaling complexes are tyrosine-specific kinases including focal adhesion kinase (FAK) and members of the Src family of cytoplasmic tyrosine kinases, as well as several other proteins including tensin, paxillin and Cas, which can be phosphorylated on tyrosine residues (Vuori, 1998; Cary and Guan, 1999). Basal tyrosine phosphorylation of focal adhesion proteins is essential for their formation and maintenance. However, increased tyrosine phosphorylation of focal adhesions, such as that which occurs in RCS cells upon FGF

signaling, can disrupt these structures, as was previously shown for cells expressing the oncogenic form of Src kinase (Rohrschneider, 1980; Volberg et al., 1991). Interestingly, the Src family of kinases is also a potential substrate for FGF receptors (Yayon et al., 1997), and activation of FAK was observed in response to FGF stimulation (Klint and Claesson-Welsh, 1999).

Upon activation of FGF signaling, FGFR3 selectively disappears from the focal adhesions, leaving vinculin behind. This ligand-receptor complex is internalized and targeted for degradation with a half-life of 30 minutes (Monsonigo-Ornan et al., 2000), which corresponds with the observed disappearance of FGFR3 from the focal adhesions within 1 hour and the decrease in the tyrosine-phosphorylation level within a similar time frame. These events are subsequently (>1 hour later) followed by dissolution of the focal adhesion microstructure that together with changes in cytoskeleton-associated proteins such as Ezrin (Tsutita and Yonemura, 1997; Bretscher, 1999) may lead to the observed reorganization of the actin network and cell rounding. We speculate that activation of FGFR3 at the substrate adhesion sites is the trigger for its removal, which may contribute to the cells' detachment from the substrate.

Multiple studies have shown that cell cycle events require signals provided by both soluble factors and the cytoskeleton and that these effects are usually restricted to the G1 phase of the cell cycle (Assoian and Zhu, 1997). In an early study, Folkman and Moscona (Folkman and Moscona, 1978) demonstrated that cell shape is tightly coupled to DNA synthesis and growth. In RCS cells, changes in FGF receptor localization were already observed 1 hour after induction with FGF9, whereas changes in cell shape and cell cycle distribution were detected later, after 6 and 10 hours, respectively. It is tempting to speculate that the cell-surface localization of the activated FGFR may determine the nature of its signal. FGFR in focal adhesions may activate growth arrest whereas extrajunctional FGFR may lead to mitogenesis. It also remains a challenge to elucidate whether growth arrest and cell shape are coupled or represent independent consequences of FGF induction.

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