JCB Article

A network of transcriptional and signaling events is activated by FGF to induce chondrocyte growth arrest and differentiation

Lisa Dailey, Emmanuel Laplantine, Riccardo Priore, and Claudio Basilico

Department of Microbiology, New York University School of Medicine, New York, NY 10016

Activating mutations in FGF receptor 3 (FGFR3) cause several human dwarfism syndromes by affecting both chondrocyte proliferation and differentiation. Using microarray and biochemical analyses of FGF-treated rat chondrosarcoma chondrocytes, we show that FGF inhibits chondrocyte proliferation by initiating multiple pathways that result in the induction of antiproliferative functions and the down-regulation of growth-promoting molecules. The initiation of growth arrest is characterized by the rapid dephosphorylation of the retinoblastoma protein (pRb) p107 and repression of a subset of E2F target genes by a mechanism that is independent of cyclin E–Cdk inhibition. In contrast, hypophosphorylation of pRb and p130 occur after growth arrest is first detected, and may contribute to its maintenance. Importantly, we also find a number of gene expression changes indicating that FGF promotes many aspects of hypertrophic differentiation, a notion supported by in situ analysis of developing growth plates from mice expressing an activated form of FGFR3. Thus, FGF may coordinate the onset of differentiation with chondrocyte growth arrest in the developing growth plate.

Introduction

Development of most skeletal elements occurs through the multistep process of endochondral ossification in which a cartilage template is converted into bone. The formation of the cartilage template involves mesenchymal condensation and chondrogenic differentiation. Reserve zone chondrocytes transit through maturational stages of proliferation, prehypertrophy, and hypertrophy within the epiphyseal growth plate, and eventually undergo apoptosis as the hypertrophic zone becomes invaded by blood vessels and osteoprogenitor cells, leading to the formation of trabecular bone.

The extent of longitudinal bone growth depends on the rates of chondrocyte proliferation and maturation, which are in turn controlled and coordinated by extensive cell–ECM interactions and a host of signaling networks elicited by FGFs, insulin, PTHrP, Indian hedgehog (Ihh), and BMPs, among others (Olsen et al., 2000; Wagner and Karsenty, 2001; Karsenty and Wagner, 2002). A key role for FGF signaling was made apparent by the discovery that gain of function mutations within FGF receptor 3 (FGFR3)* cause several forms of human dwarfism including achondroplasia, hypochondroplasia, and thanathophoric dysplasia, which are all characterized by the reduced growth of long bones (Ornitz and Marie, 2002). Subsequent works in tissue culture and in mouse models of both gain and loss of function mutations in FGFR3 indicated that FGF signaling restrains chondrocyte proliferation and possibly differentiation by direct action on chondrocytes, as well as by indirect mechanisms (Ornitz and Marie, 2002). However, the notion that FGF signaling inhibits chondrocyte differentiation has recently been challenged by Minina et al. (2002).

The growth arrest exhibited by chondrocytes is in stark contrast to the typical stimulatory response to FGF exhibited by most other cell types, and appears to result from a unique downstream response of proliferating chondrocytes to FGF rather than a novel signaling property of FGFR3 (Wang et al., 2001). In vivo analyses have shown increased expression of STAT proteins, p21, and Ink family Cdk inhibitors (CDKIs)

The online version of this article includes supplemental material.

Address correspondence to C. Basilico, Dept. of Microbiology, NYU School of Medicine, 550 First Avenue, New York, NY 10016. Tel.: (212) 263-5341. Fax: (212) 263-8714. E-mail: basilc01@med.nyu.edu; or L. Dailey, Dept. of Microbiology, NYU School of Medicine, 550 First Avenue, New York, NY 10016. Tel.: (212) 263-5341. Fax: (212) 263-8714. E-mail: dailel01@med.nyu.edu

Key words: DNA; microarrays; E2F; retinoblastoma proteins; Cdk

*Abbreviations used in this paper: CDKI, Cdk inhibitor; FGFR3, FGF receptor 3; Ihh, Indian hedgehog; MMP13, matrix metalloproteinase 13; OPG, osteoprotegerin; OPN, osteopontin; pRb, retinoblastoma protein; RCS, rat chondrosarcoma.

The Journal of Cell Biology, Volume 161, Number 6, June 23, 2003 1053–1066
http://www.jcb.org/cgi/doi/10.1083/jcb.200302075

The Journal of Cell Biology

in response to excessive FGF signaling, whereas other reports have demonstrated that FGF-mediated inhibition requires STAT1 function, both in vitro and in vivo (Chen et al., 1999; Li et al., 1999; Sahni et al., 1999, 2001; Aikawa et al., 2001). However, neither p21- nor STAT1-null mice exhibit overt skeletal abnormalities (Brugarolas et al., 1995; Durbin et al., 1996). Analyses of mice in which genes for members of the pRb family had been inactivated showed that the absence of both p107 and p130 impaired endochondral bone development by causing excessive proliferation and decreased differentiation of growth plate chondrocytes (Cobrinik et al., 1996; Rossi et al., 2002). In line with this observation, we established that FGF-mediated growth arrest of chondrocytes requires functional p107 and p130 proteins, but not retinoblastoma protein (pRb), and that FGF treatment of cultured chondrocytes causes the hypophosphorylation of all three pRbs (Laplantine et al., 2002). Although p107 appears to play the major role, p130 is also required for maximum growth inhibition (Laplantine et al., 2002). Together, these observations suggest that STAT1 mediates some aspects of FGF signaling, but that FGF may use additional components to affect chondrocyte proliferation and/or differentiation.

To gain further insight into the mechanisms by which FGF signaling affects these processes, we have used genome wide expression profiling to elucidate the cascades of gene expression after FGF treatment of a cultured rat chondrosarcoma (RCS) chondrocytic cell line. Our results support a model in which FGF inhibition of the chondrocyte cell cycle is achieved through the activation of multiple pathways that act via a "two step" mechanism: (1) direct signaling to negatively regulate the activities or transcription of key cell cycle components and mediators of signals that stimulate chondrocyte proliferation ("initiation of growth arrest"); and (2) Cdk inhibition and the eventual transcriptional down-regulation of additional cell cycle protein genes ("maintenance of growth arrest"). We also find dramatic changes in expression of many genes associated with chondrocyte differentiation, consistent with the notion that FGF initiates several aspects of hypertrophic differentiation. These data have been supported by in situ analyses of growth plates from mice harboring an activated FGFR3. Together, these results portray several mechanisms by which excessive FGF signaling contributes to growth plate pathologies, but also provide suggestions regarding the role of FGF in normal bone development.

Results

The kinetics of FGF-induced cell cycle arrest in RCS cells

The RCS cell line exhibits many properties of proliferating chondrocytes, including the expression of collagen II and FGFR3 (Mukhopadhyay et al., 1995). Previous work has shown that treatment of RCS cells with FGF1 causes an inhibition of cell cycle progression (Sahni et al., 1999). To correlate gene expression changes with the kinetics of this growth arrest, we performed a FACScan™ analysis of RCS cells at various intervals after treatment with FGF1 and heparin in the presence of 10% serum. 3 and 6 h of FGF treatment caused an initial increase in the number of cells

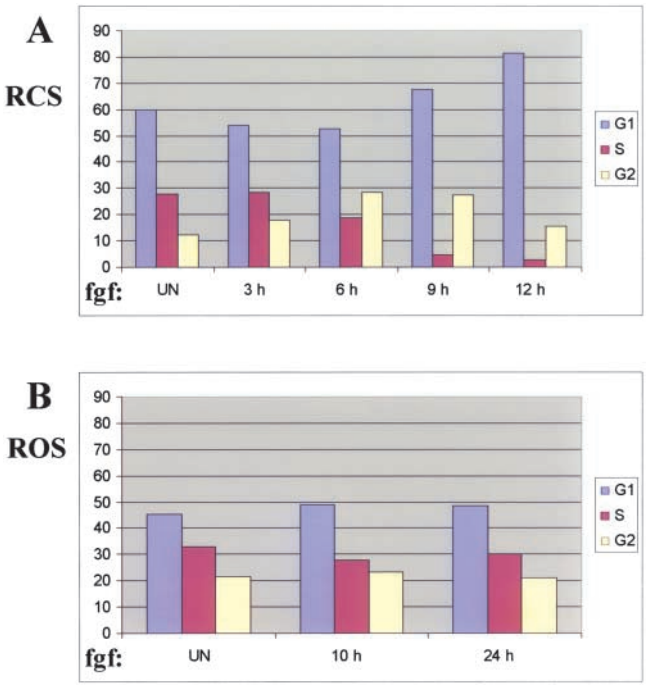

Figure 1. **FACScan™ analysis of RCS and ROS cells in response to FGF treatment.** Growing cultures were treated with 5 ng/ml FGF1 and 10 mg/ml heparin for the indicated times and subjected to flow cytometry. UN indicates RCS (A) or ROS (B) cells treated with heparin only. Numbers on the y axis indicate percentage of total cells analyzed per sample.

in G2, consistent with previous reports (Aikawa et al., 2001; Rozenblatt-Rosen et al., 2002; Fig. 1 A). Because of the G2 block, the percentage of cells in G1 declined relative to the untreated samples at 3 h as the G1 cells proceeded to S phase, indicating that most of the cells were not yet blocked in G1. However, by 6 h of treatment, cells from G1 were no longer entering the S phase. Thus, the G1 block was first clearly manifest by 6 h after FGF addition. By 9 h, the G2 block had been relieved and cells continued to accumulate in G1. These results demonstrate that FGF treatment of RCS cells causes a transient G2 block that is evident by 3 h but relieved by 6–9 h, and a sustained G1 block first clearly evident after 6 h of FGF treatment. For comparison, we used the osteoblastic cell line ROS 17/2.8, whose proliferation was not inhibited by FGF (Fig. 1 B), despite the fact that they also harbor functional FGFR3 (Mansukhani et al., 2000).

Overview of the gene expression profile of FGF-treated RCS

RNA was prepared from RCS cells after 1, 3, 6, 10, and 24 h treatment with FGF1 and heparin or with heparin only. To identify changes in gene expression that required new protein synthesis, RNA was also prepared from cells treated with FGF in the presence of cycloheximide. To assist in the identification of genes that might be relevant to FGF-induced growth arrest in RCS cells, RNA was prepared at the same time points from FGF-treated ROS cells. After conversion into biotinylated cRNA, the samples were hy-

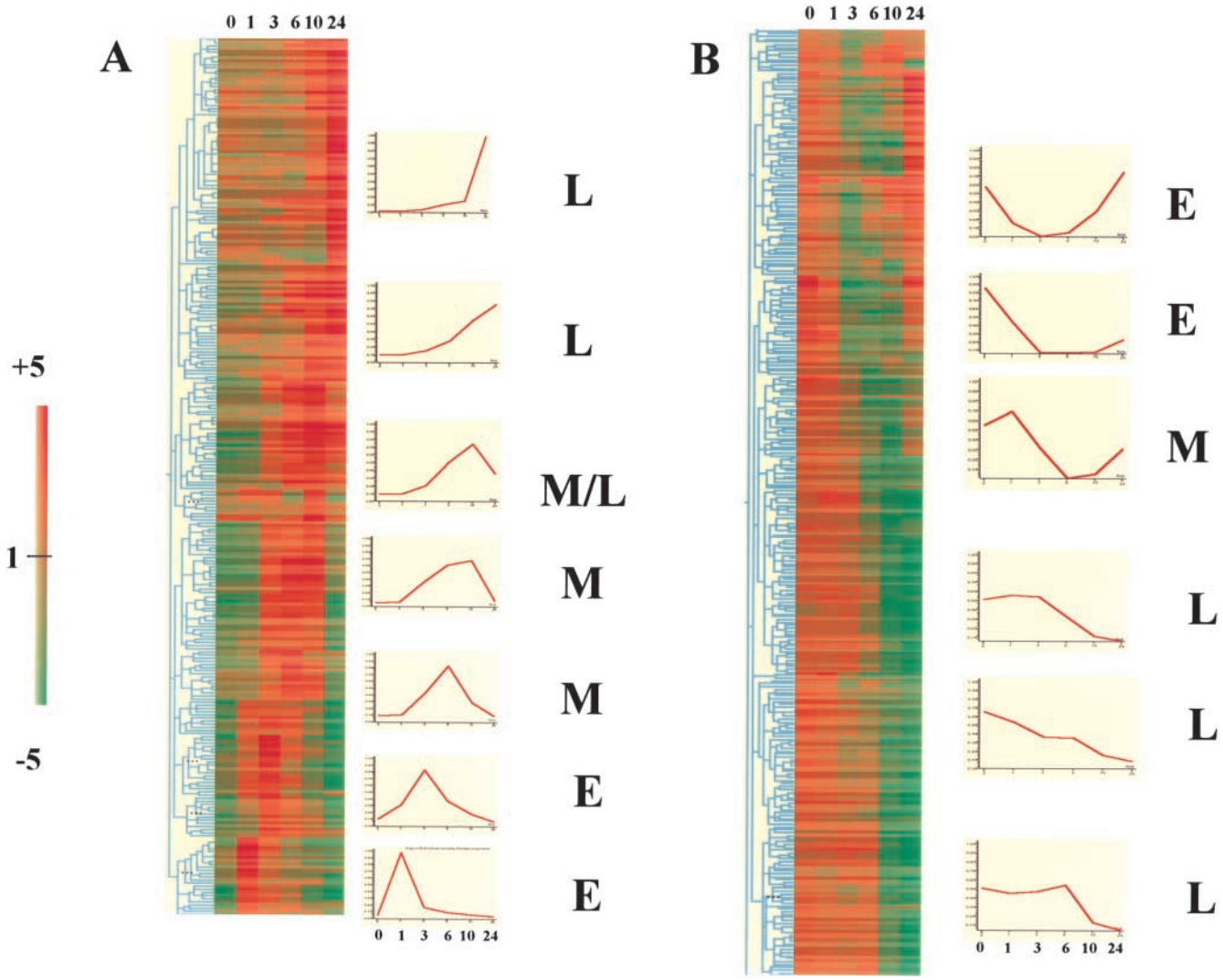

Figure 2. **Gene expression profiles after FGF treatment of RCS cells.** RNA samples prepared from RCS cells after 0, 1, 3, 6, 10, or 24 h of FGF treatment were converted into biotinylated cRNAs and hybridized to rat microarrays (Affymetrix). Genes up-regulated (A) or down-regulated (B) by greater than threefold with respect to untreated control samples were subjected to hierarchical clustering as detailed in Materials and methods. Average expression is defined by the GeneSpring® software (Silicon Genetics) and assigned a value of 1, whereas values greater or less than this value are visualized as more intense shades of red or green, respectively. Individual genes are represented in rows from left to right, and their relative expression level is depicted according to the colorimetric scale at each time point indicated above the dendrograms. Major patterns within the dendrograms were determined for selected nodes and are displayed to the right of each dendrogram. By relating each of these expression profiles to changes in the cell cycle as defined by the FACScan™ analysis, genes within these subgroups were defined as early (E), mid-(M), or late (L) response genes.

bridized to DNA oligonucleotide microarrays representing 8,799 rat cDNAs and ESTs. No significant change was observed in the expression of RNAs derived from the heparin-treated control cultures. In contrast, the expression of many genes was either induced (280) or repressed (697) by greater than threefold during the course of FGF treatment.

To correlate the regulation of specific gene subsets with changes in the cell cycle, the genes were grouped according to their expression patterns using hierarchical clustering to create the dendrogram shown in Fig. 2, and these patterns were classified as representative of "early response genes," "mid-response genes," or "late response genes." A partial list of the genes contained in these subgroups is presented in Table I. The gene array results were verified using Northern analysis of a selection of genes (Fig. 3).

Early response genes

This group represents genes exhibiting changes in expression between 0–3 h after FGF addition, and thus preceding or coinciding with G2 arrest. (Table I; Fig. 2, E subgroups). Included within this group are genes for many transcription factors that are classically defined as "immediate early proteins," such as members of the AP-1 complex (c-jun, Jun B, c-fos), ets1, ATF 3, and Egr-1 (not depicted). As expected, the induction of most of these genes was not inhibited by cycloheximide. Although many of these genes were also induced in ROS cells, the induction was generally less robust and/or occurred with distinct kinetics from that in RCS (e.g., Fra-1).

The expression of several genes that would be expected to have a distinct bearing on the cell cycle was specifically al-

Table I. **Compilation of genes regulated by FGF treatment of RCS cells and comparison with their expression in ROS cells**

		RCS						ROS				
		1	3	6	10	24		1	3	6	10	24
	GenBank/EMBL/DDBJ accession no.						CHX					
Early response genes												
Transcription												
Krox-24	U75397	**119**	**85**	**50**	**14**	-	#	**31**	**16**	**9.3**	2.6	0.2
krox20	U78102	**17**	**11**	**22**	**15**	**8.8**		**14**	**6.3**	2.4	2.3	0.8
HES-1	D13417	**7.2**	1.6	-	-	-	C	1.8	1.7	2.1	2.6	1.5
Fra-1	M19651	**111**	**336**	**187**	**66**	-		1.8	**3.9**	3.1	2.7	0.8
fra-2	U18982	1.4	**3.2**	2.2	-	-		1	0.5	0.7	0.8	1
c-fos	X06769	**8.2**	1.6	-	-	-		**6.3**	0.6	0.5	0.7	1.1
v-jun homologue	AA945867	**4.6**	**3.4**	-	1.6	-		1.2	0.7	1	0.6	0.5
c-jun	X17163	**4**	**3.7**	**3.9**	2.9	-		1.4	0.6	1.2	0.7	0.8
Jun B	AA891041	**18**	**8.4**	**10**	-	-		**3.1**	2.2	2.3	1.3	1.9
GADD153	U30186	**5.1**	**3.6**	1.4	1	0.9		1.1	0.8	0.6	0.7	**3.3**
ATF 3	M63282	**13**	**2.8**	0.7	0.9	1.5		2.1	1.1	0.7	0.7	**3.4**
Ets-1	L20681	1.1	**3.8**	2.6	1.3	-	#	1	1.6	2.7	1	0.8
Dlx-3	D31734	0.3	-	-	-	0.3		0.7	0.4	0.4	0.5	0.9
Signal transduction												
MKP-1	S74351	**71**	**8.4**	**3.3**	-	-		1.8	0.2	0.3	0.4	1
MKP-3	U42627	**6**	**105**	**111**	**100**	-	C	**15**	**34**	**27**	**27**	0.5
uPAR-1	X71898	2.8	**6.7**	**4.9**	**4.1**	**3.1**	#	**3.9**	**3.9**	2.8	2	0.9
PTHrPR	AB012944	0.7	0.3	0.1	0.2	0.7		0.4	0.5	0.3	0.3	0.6
fzd	L02529	0.7	-	-	0.3	0.9		0.9	0.3	0.5	0.5	0.7
ERK1	M61177	0.9	0.6	0.2	0.4	1.2	#	0.9	0.8	1.1	1	1
P38 MAPK	AI171630	0.8	0.1	0.2	-	0.3	C	0.8	0.4	0.7	0.6	0.9
IRS-1	X58375	0.4	0.1	0.1	-	0.2	C	0.9	0.3	0.3	0.3	0.5
Cell cycle												
PC3	M60921	**25**	2.1	0.8	-	-		**4.9**	2.1	1.3	0.4	0.8
p21	U24174	2.1	**3.3**	1.3	0.8	-		1.4	2	2.3	1.3	1.3
GADD45	L32591	**4.3**	**3**	2.8	1.8	-	#	0.7	0.5	0.9	1	2.5
Pold1	AJ222691	0.8	-	-	-	-		0.9	0.6	0.8	0.7	0.8
Pola2	AJ011606	0.9	0.3	0.5	0.4	0.5		0.9	0.5	0.6	0.9	1
Id 1	L23148	0.4	-	-	0.2	-		0.5	0.3	1.2	2.3	2.2
Id 2	AI230256	0.5	-	-	-	0.1		1.8	0.3	0.8	1.6	1.3
Id 3	AF000942	0.4	-	-	-	-		1.2	0.7	1.1	1.6	1.8
Mid-response genes												
Transcription												
HMG I (Y)	X62875	1.3	**3.7**	**3.7**	**3.8**	**3.1**	C	0.7	**4.7**	**10**	**10**	**12**
Hnf3b	L09647	0.8	**4.4**	**7.4**	**4.8**	1.9	C	-	-	-	-	-
Signal transduction												
Jag 1	AA900503	1.4	**3.1**	**4.2**	2.9	1.6	C	1.4	1.6	1.5	12	0.9
Ephrin A1	AA892417	1.4	**4**	**9.5**	**6.7**	**5.7**	C	1.2	0.7	0.9	1	1.2
BMP3b	D49494	0.8	0.5	-	-	0.2		-	-	-	-	-
Cell cycle												
p16/Ink4	S79760	1.2	**2.8**	**3.3**	**3.6**	-		-	-	-	-	-
Cyclin D1	D14014	1.2	**6.9**	**14**	**14**	**5.9**	C	0.9	1.3	1.2	1.6	1.1
Differentiation-related												
DEC1/bhlh2	AF009330	2.1	**3.2**	**3.2**	2.8	2.2	#	1.1	1.5	1.6	1	1.1
MMP13	M60616	1.9	**35**	**74**	**108**	-	C	0.5	1.3	0.5	0.6	0.6
OPG	U94330	1.2	**3.2**	**4.1**	**3.7**	1.6	C	1.4	1.1	0.6	0.8	0.8
TIMP-1	AI169327	-	**184**	**321**	**377**	**999**	C	1	1.5	1.1	1.4	1.1
OPN	M14656	1.3	**12**	**23**	**31**	**214**	C	1	2.1	2.8	**3.2**	**3.3**
Late response genes												
Transcription												
STAT5a1	U24175	-	-	-	-	**4.2**	#	-	-	-	-	-
c-Myc	Y00396	1.4	1.2	1	0.2	0.3	#	2.3	**3.2**	**3.6**	2.4	1.1

Table I. **Compilation of genes regulated by FGF treatment of RCS cells and comparison with their expression in ROS cells (Continued)**

Signal transduction												
FGFR-1	D12498	1	1.4	**3.1**	**3.6**	2.4	C	1.1	0.7	1.4	1.6	1.2
ERK3	M64301	1.1	0.9	0.8	1	-	C	1	0.9	1.2	0.9	1
Cell cycle												
Cyclin E	D14015	1	0.9	0.7	0.3	0.4	#	0.8	0.8	0.9	1	0.7
Cyclin B1	AA998164	1.1	0.9	1.2	0.7	-	#	0.9	0.8	0.9	0.8	1.1
Cdk2-alpha	D28753	0.9	0.8	0.7	-	-	#	0.6	0.5	0.7	1	1.1
Cdk2-beta	D28754	1	0.6	0.5	-	-	#	0.9	0.4	0.8	-	0.8
p55cdc	AF052695	0.9	0.6	0.9	0.6	-	#	0.8	0.8	0.7	0.8	1.1
cdc2	AB005540	0.9	1.5	1	0.8	-	#	0.7	1.6	1.8	0.8	1.1
CDC5	AF000578	1	0.8	0.5	0.5	0.3	C	0.7	0.7	0.8	0.9	1
CDC25B	D16237	0.9	1	1	0.9	0.3	#	1	0.8	1	0.8	1.2
CAK1	X83579	1.2	1.3	1.7	0.5	-	#	1	0.9	1	1	0.9
Dpola3	AJ011607	0.9	0.6	0.6	0.3	0.2	C	0.9	0.8	0.7	0.8	0.8
Dpola4	AJ011608	1	1	0.6	0.3	0.2	C	0.9	0.8	1	0.9	1.1
Dpola1	AJ011605	0.8	0.7	0.5	0.1	-	C	0.8	0.4	0.8	0.6	1.1
PCNA	M24604	1.2	1.4	0.9	0.4	0.3	#	1.2	1	1	1	1
Tyms	L12138	0.9	0.8	0.7	-	-	C	0.9	0.8	0.8	0.8	1
dUTPase	U64030	0.9	0.9	0.7	0.6	0.1	C	0.9	0.8	0.9	0.7	0.9
PTTG	U73030	1.2	1	0.9	1.1	-	#	0.9	1	0.9	0.8	1.1
TOP2A	AA899854	1	1.1	1.1	0.4	-	#	0.9	0.8	0.9	0.7	0.9
MSH2	X93591	0.8	0.8	1	0.3	0.3	#	0.7	0.6	0.7	0.7	1.1
ODC	J04791	0.9	1	0.9	0.5	-	#	1.1	1	1.1	1.2	1.1
Differentiation-related												
Annexin V	D42137	0.9	1.7	1.8	**3**	**3.4**	C	1	1.4	1.2	1	1
Annexin II	AA946503	-	**4.1**	**12**	**16**	**3.4**	C	-	-	-	-	-
Chad	AF004953	0.7	0.7	0.6	0.8	**6**	#	-	-	-	-	-
TIMP-2	S72594	1.1	1.1	1.2	0.7	**3.1**	#	1	0.7	0.8	0.9	0.8
SGP-1	S81353	1.1	1.2	1.4	1.7	**4.1**	#	0.8	0.7	0.9	1.2	1

Placement of genes into the early, mid-, or late response list was based on the clustering analysis of Fig. 2 as explained in the text. 1, 3, 6, 10, and 24 at the top of the columns indicate hours of FGF treatment. Numbers represent fold induction as compared with the expression of each gene in untreated cells. Induction or down-regulation of expression by threefold or greater is shown as boldface or underlined, respectively. Dashes indicate no expression. Data obtained from analysis of RCS cells treated with FGF in the presence of cycloheximide are summarized in the column CHX, where C indicates expression changes inhibited by cycloheximide, and blank spaces depict those unaffected by cycloheximide; # indicates data that were ambiguous because cycloheximide itself altered the mRNA level or because the change in gene expression occurred after 6 h of the cycloheximide experiment. MKP, map kinase phosphatase; uPAR, urinary plasminogen activator; PTHrPR, parathyroid hormone–related peptide receptor; fzd, frizzled; IRS, insulin receptor substrate; Pold, DNA polymerase; Jag, jagged; TIMP, tissue inhibitor of metalloproteinase.

tered in RCS cells during the early response stage (Table I). For example, the gene for PC3, a member of the tob family that is thought to play an antiproliferative role (Tirone, 2001), was strongly induced during the first hour of FGF treatment. We also noted the down-regulation of gene expression of two DNA polymerase (Pol) subunits that occurred as a direct result of FGF signaling because it was also observed in the presence of cycloheximide. The genes for p21 and GADD45 were also specifically induced in RCS cells. Because both p21 and GADD45 can inhibit cyclin/Cdk activities (Sherr and Roberts, 1999; Zhan et al., 1999; Vairapandi et al., 2002), their induction may contribute to the transient G2 arrest of RCS cells seen by 3 h.

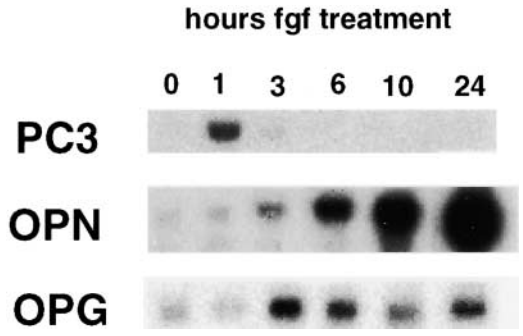

Figure 3. **Confirmation of the microarray results using Northern analysis.** RNA was isolated from RCS cells treated with FGF for the indicated times and subjected to Northern analysis using radiolabeled probes for PC3, osteopontin (OPN), or osteoprotegerin (OPG) as described in Materials and methods.

An additional feature of the early response of RCS cells to FGF was the immediate and precipitous decline in expression of all three Id protein mRNAs (Table I). The Id proteins act as dominant-negative transcriptional inhibitors of bHLH factors (Yokota and Mori, 2002), and can also promote cell proliferation by antagonizing the activities or expression of key cell cycle regulators including pRbs and the CDKIs p21 and Ink4a/p16 (Yokota and Mori, 2002). In addition, we noted the down-regulation of several other genes that play a role in chondrocyte proliferation, including the signal transduction components PTHrP receptor, Wnt receptor (frizzled), and the insulin receptor substrate IRS-1 (Table I). Together, analysis of the early response genes re-

veals specific expression patterns of transcription factors and signaling molecules that already distinguish RCS and ROS cell responses to FGF, including the activation of genes encoding antiproliferative functions (GADD45, PC3, p21, and jun B), and the dramatic down-regulation of several growth-promoting signaling molecules.

Mid-response genes

Genes in this group exhibit a significant change in expression between 3 and 6 h after FGF treatment, and thus precede or coincide with the G1 arrest observed at 6 h (Table I; Fig. 2, subgroup M). Most notable among these with regard to cell cycle regulation was induction of the Ink4a/p16 gene. p16 is an inhibitor of the G1 cyclin D–Cdk complex and an important mediator of cell cycle arrest (Sherr and Roberts, 1999). Induction of the p16 gene was not inhibited by cycloheximide, was not observed in ROS cells, and was sustained through 10 h of FGF treatment (Table I), consistent with the notion that p16 also plays a role in the FGF-mediated growth arrest of RCS cells. Paradoxically, we also noted a strong and sustained induction of cyclin D1 mRNA. Although cyclin D1 induction is usually associated with mitogenic responses, it has also been observed before the FGF- or NGF-induced differentiation of growth-arrested neuronal PC12 cells (Yan and Ziff, 1995).

In addition to changes in expression of these cell cycle regulators, we also noted a dramatic increase in the expression of several genes normally elevated in differentiated hypertrophic chondrocytes. These include DEC1 (Shen et al., 2002), osteopontin (OPN), and several genes encoding components or modifiers of the ECM such as those for the collagen matrix metalloproteinase 13 (MMP13) and TIMP-1. Thus, in the mid-response stage, the cells became arrested in G1 and the expression of molecules typical of differentiated chondrocytes was induced.

Late response genes

Late response genes exhibited significant changes in expression just before or during 10–24 h after FGF treatment, when the cells continue to accumulate in G1 (Fig. 1). These genes included those within subgroup L in Fig. 2 as well as genes down-regulated only at 24 h and not included in the

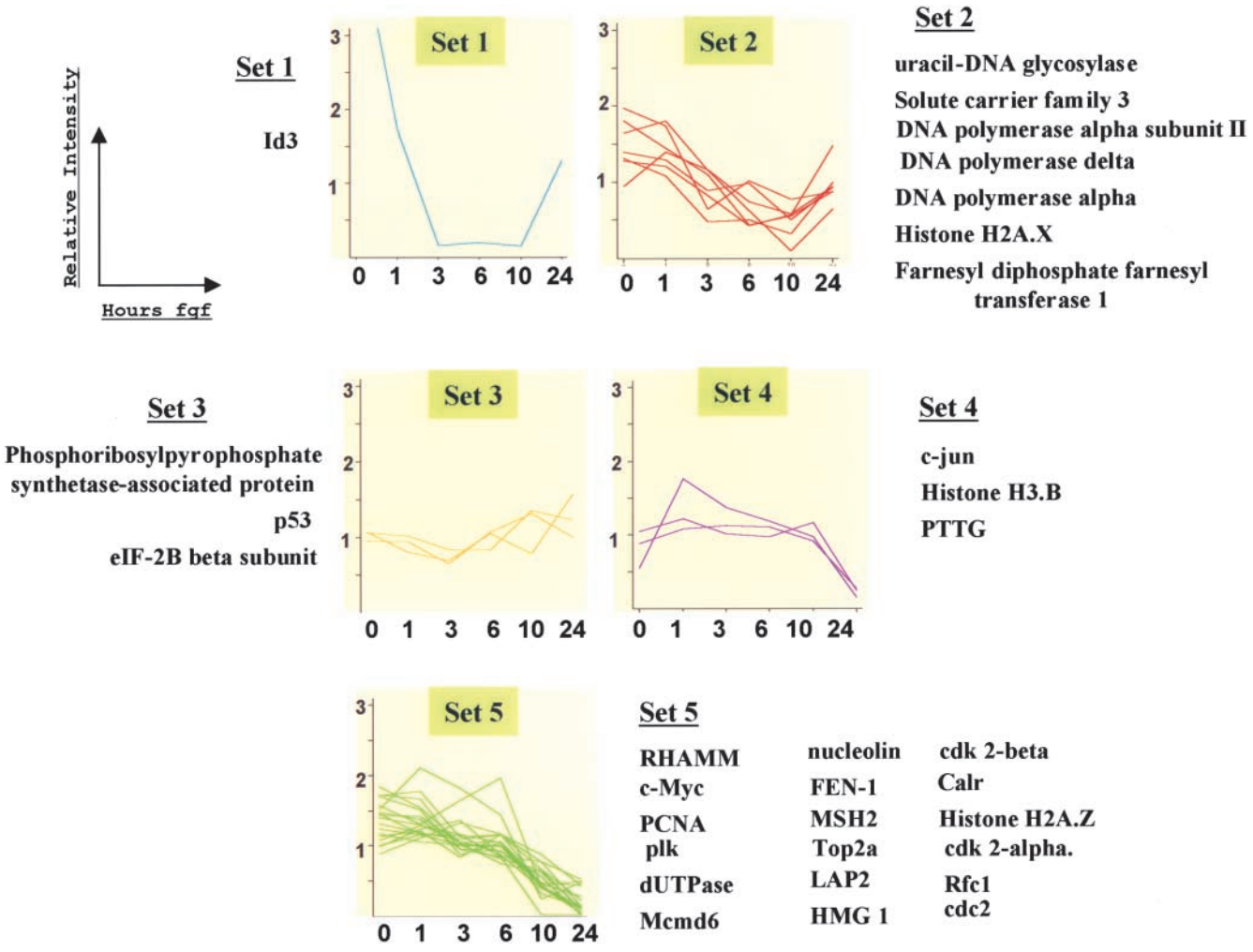

Figure 4. **E2F target genes are down-regulated in RCS cells in response to FGF.** The expression profiles of rat homologues of previously identified human E2F target genes (Ren et al., 2002) were subjected to k-means clustering as detailed in Materials and methods. Each line within the graphs represents the relative expression profile for one of the genes listed.

dendrogram. Notably, we observed a nearly universal down-regulation in the expression of many major cell cycle regulators, including that of cyclins E and B1, Cdk2, cdc2, CDC5, CDC25B, and CAK1 (Table I). Furthermore, several components of DNA synthesis were down-regulated, including ODC, additional DNA polymerase subunits, and PCNA. Importantly, these changes occurred exclusively in the FGF-treated RCS samples and were not observed in either untreated RCS cells or in ROS cells. These results indicate that transcription of many of the components of the cell cycle machinery had become essentially shut down by 10–24 h, and may represent a mechanism for cell cycle arrest that is distinct from (or a consequence of) that observed at 6 h after FGF addition. In addition to the continued production of the ECM molecules initiated during the mid-response stage, we also noted the induction of mRNAs for several fibronectin isoforms, the cell surface protein CD14, annexin V, TIMP-2, sulphated glycoprotein-1, dynamin-1, and chondroadherin at these later time points (Table I and unpublished data).

Expression of E2F target genes in response to FGF signaling

Previous reports have underscored an essential role for the Rb-related p107 and p130 proteins in chondrocyte growth arrest and bone development (Cobrinik et al., 1996; Rossi et al., 2002). These pRbs become hypophosphorylated after FGF treatment of RCS cells (Laplantine et al., 2002). Hypophosphorylated pRbs mediate growth arrest primarily by binding E2F transcription factors, leading to either the unavailability of free, activating E2F1–3 factors and/or the recruitment of transcriptionally repressive p107– or p130–E2F4/5 complexes to E2F target genes (Trimarchi and Lees, 2002). Using a combination of chromatin immunoprecipitation and microarray technology, a number of putative human E2F target genes have been identified whose transcription is repressed in serum-starved fibroblasts (Ren et al., 2002). These genes include a number of essential components of cell cycle regulation, and DNA synthesis and repair. We analyzed the expression of some of these E2F target genes in RCS cells to ascertain whether we could correlate pRb/p107/p130 hypophosphorylation with down-regulation of E2F target genes, and whether repression of these genes occurred before or subsequent to the G1 arrest. As shown in Fig. 4, the majority of these genes were down-regulated with either relatively rapid (sets 1 or 2) or delayed (sets 4 and 5) kinetics, whereas a few genes exhibited no change in their mRNA levels (set 3) after FGF treatment. Notably, none of these genes was significantly induced with the exception of the early, transient activation of c-jun, and none were down-regulated in FGF-treated ROS cells (unpublished data). Genes within sets 1 and 2 comprised genes included in the early or mid-response genes (Table I), and therefore were down-regulated before G1 arrest. These included Id3 (set 1) and the DNA polymerase subunits δ and α II (set 2). Importantly, the down-regulation of several of the E2F target genes in these groups was observed in the presence of cycloheximide. In contrast, most of the mRNAs within sets 4 and 5 declined after the G1 arrest at 6 h. and their down-regulation was sensitive to cycloheximide (Fig. 4; Table I). Thus, transcriptional repression of the E2F target genes in sets 1 and 2 occurred before, and not as a consequence of, G1 arrest, probably while cells were still traversing the S phase. These results are consistent with the established central role of the p107/p130 proteins in chondrocyte growth arrest and strongly implicate a repression of a subset of critical E2F target genes in the initiation of growth arrest by FGF.

The Rb family proteins exhibit distinct kinetics of dephosphorylation

Western analysis showed that proliferating RCS cells contained predominantly hyperphosphorylated forms of pRb and

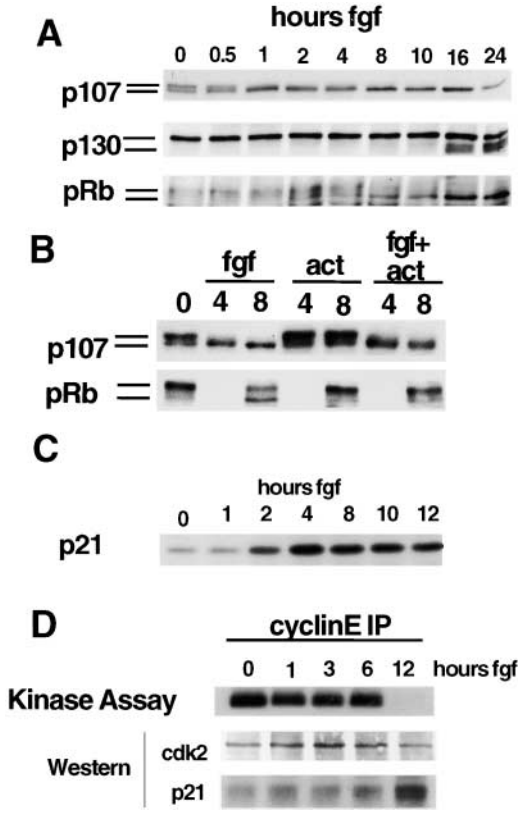

Figure 5. **Hypophosphorylation of the pRbs occurs with different kinetics and by distinct mechanisms in response to FGF.** (A) Kinetics of p107, p130, and pRb dephosphorylation. Total protein lysates were prepared from RCS cells at the times of FGF treatment indicated and subjected to Western analysis using the antibodies indicated. Bars to the left of the image depict the position of the hyperphosphorylated (top) or hypophosphorylated (bottom) forms of each protein. (B) RCS cells were treated with FGF in the presence or absence of actinomycin D, and whole-cell extracts were subjected to Western analysis using p107 or pRb antibodies. Samples derived from cells treated with FGF (fgf), acinomycin D (act), or both (fgf+act) are indicated above the lanes. (C) Western analysis of p21 expression. Protein extracts from FGF-treated RCS cells were subjected to Western analysis using anti-p21 antibody. (D) Kinetics of cyclin E–Cdk inhibition and association with p21. Cyclin E–Cdk2 complexes were immunoprecipitated from protein extracts of RCS cells after treatment with FGF for the times indicated. Kinase activity was assessed in vitro in the presence of $\gamma[^{32}P]$ATP and histone H1 substrate as detailed in Materials and methods. The remainder of the immunoprecipitate was used for Western analysis using antibodies against Cdk2 or p21.

p130 and a mixture of hyper- and hypo-phosphorylated p107 proteins (Fig. 5 A). On FGF treatment, p107 underwent rapid dephosphorylation within the first hour. In contrast, the hypophosphorylated form of pRb predominated only after 8 h, and those of p130 were first observed after 16 h of FGF treatment (Fig. 5 A). These distinct kinetics indicated that the hypophosphorylated forms of the pRbs may be generated by different mechanisms. Therefore, dephosphorylation of p107 and pRb by FGF was compared in the presence or absence of the transcription inhibitor actinomycin D. Hypophosphorylated p107 was still observed when the cells were treated with FGF in the presence of actinomycin (Fig. 5 B) or cycloheximide (unpublished data), indicating that this process does not require new gene transcription or protein synthesis. In contrast, actinomycin abolished the ability of FGF to cause the hypophosphorylation of pRb. Together, these observations are consistent with the notion that dephosphorylation of p107 is regulated directly and rapidly by FGF signaling, whereas that of pRb (and p130, unpublished data) occurs later and requires the induction of gene transcription.

Hypophosphorylation of pRb and p130 (but not p107) correlates with the inhibition of cyclin E–Cdk2 activity

In the normal cell cycle, phosphorylation of the pRbs is mediated by the G1 cyclin D–Cdk4/6 and cyclin E–Cdk2 complexes. Harbour and Dean (2000) and Aikawa et al. (2001) previously showed that cyclin E–Cdk2 (but not cyclin D–Cdk) activity is inactivated by p21 after 12 h of FGF treatment of RCS cells. However, the FGF-induced dephosphorylation of p107 that we observed in the presence of actinomycin occurred independently of p21 (or p16) gene induction (Table I and unpublished data), suggesting that p107 dephosphorylation does not result from inhibition of cyclin E–Cdk2.

To test this hypothesis, we analyzed the kinase activity of the cyclin E–Cdk2 complex and its association with p21 in RCS cells treated with FGF for 1, 3, 6, and 12 h. Western analysis indicated that the p21 protein was induced by 2 h after FGF treatment, consistent with the microarray data of Table I, and remained relatively high during the 12 h examined (Fig. 5 C). However, an in vitro kinase assay of cyclin E–Cdk2 complexes that had been immunoprecipitated from protein extracts of FGF-treated RCS cells showed that the kinase activity remained robust during the first 6 h, and was only clearly inhibited 12 h after FGF addition (Fig. 5 D). The inhibition of kinase activity correlated with a clear increase in the association of p21 with the cyclin–Cdk2 complex at 12 h (Fig. 5 D). Thus, the dephosphorylation of p107 caused by FGF treatment occurs in the presence of active cyclin E–Cdk2, and is observed before the induction of p21 and p21-mediated inhibition of cyclin E–Cdk2. These observations are consistent with the notion that FGF activates a signaling pathway that acts directly to dephosphorylate p107, leading to the assembly of p107–E2F complexes and the early repression of a subset of E2F target genes, to initiate growth arrest. In contrast, detection of the hypophosphorylated forms of pRb and p130 correlates with the kinetics of cyclin E–Cdk2 inhibition, and thus may result from p21-mediated inhibition of cyclin E–Cdk2 several hours after FGF addition.

Table II. **Gene expression changes associated with chondrocyte differentiation**

Gene product	Growth plate in vivo[a] (hypertrophic chondrocytes)	FGF-treated RCS
PTHrP receptor	Down	Down
DEC1	Up	Up
MMP13	Up	Up
OPG	Up	Up
OPN	Up	Up
FGFR1	Up	Up
ColX	Up	NC
ColII	Down	NC
Id1	Down	Down
Id2	NT	Down
Id3	Down	Down
TIMP-1	Up	Up
Annexin V	Up	Up

[a]Data derived from Fig. 6 or literature cited within the text.
NC, no change; NT, not tested.

FGF signaling promotes chondrocyte differentiation

In addition to negative regulation of the chondrocyte cell cycle, FGF signaling exerts a profound affect on the maturation of the growth plate. In contrast to the generally held interpretation that FGF inhibits chondrocyte differentiation, we have observed changes in the expression of a number of genes that are more consistent with the opposing view that FGF promotes at least some aspects of hypertrophic differentiation (Table II). These include the induction of genes previously reported to be associated with hypertrophic differentiation such as MMP13, OPN, osteoprotegerin (OPG), annexin V, and FGFR1, and the down-regulation of the PTHrP receptor (Deng et al., 1996; Minina et al., 2002). Although the expression pattern of the Id proteins has not been previously analyzed in the growth plate, their down-regulation is also a general feature of many differentiation programs (Norton et al., 1998). In contrast, we did not observe induction of expression of collagen X, the classical marker of terminally differentiated chondrocytes. This is not due to a peculiarity of the RCS cell line because we were also unable to detect collagen X gene induction in FGF-treated primary chondrocyte cultures by RT-PCR, even though induction of MMP13 and OPN could be observed (unpublished data). These data are consistent with the notion that FGF promotes several (but not all) aspects of hypertrophic differentiation.

In situ analysis was used to assess the expression of several of the genes presented in Table II in bone sections derived from P15 wild-type mice or those derived from mice homozygous for an activating mutation in FGFR3 (Gly369Cys). These mutant mice have been shown to have a chondrodysplastic phenotype with shorter long bones, disorganization of the growth plate, and reduced chondrocyte proliferation that is associated with increased expression of STAT proteins p16 and p19 (Chen et al., 1999). In situ hybridization was performed using ^{35}S-labeled antisense RNA probes corresponding to the collagen X, Id1, Id3, OPN, or Ihh genes (Fig. 6). In wild-type growth plates, Ihh expression was detected predominantly in prehy-

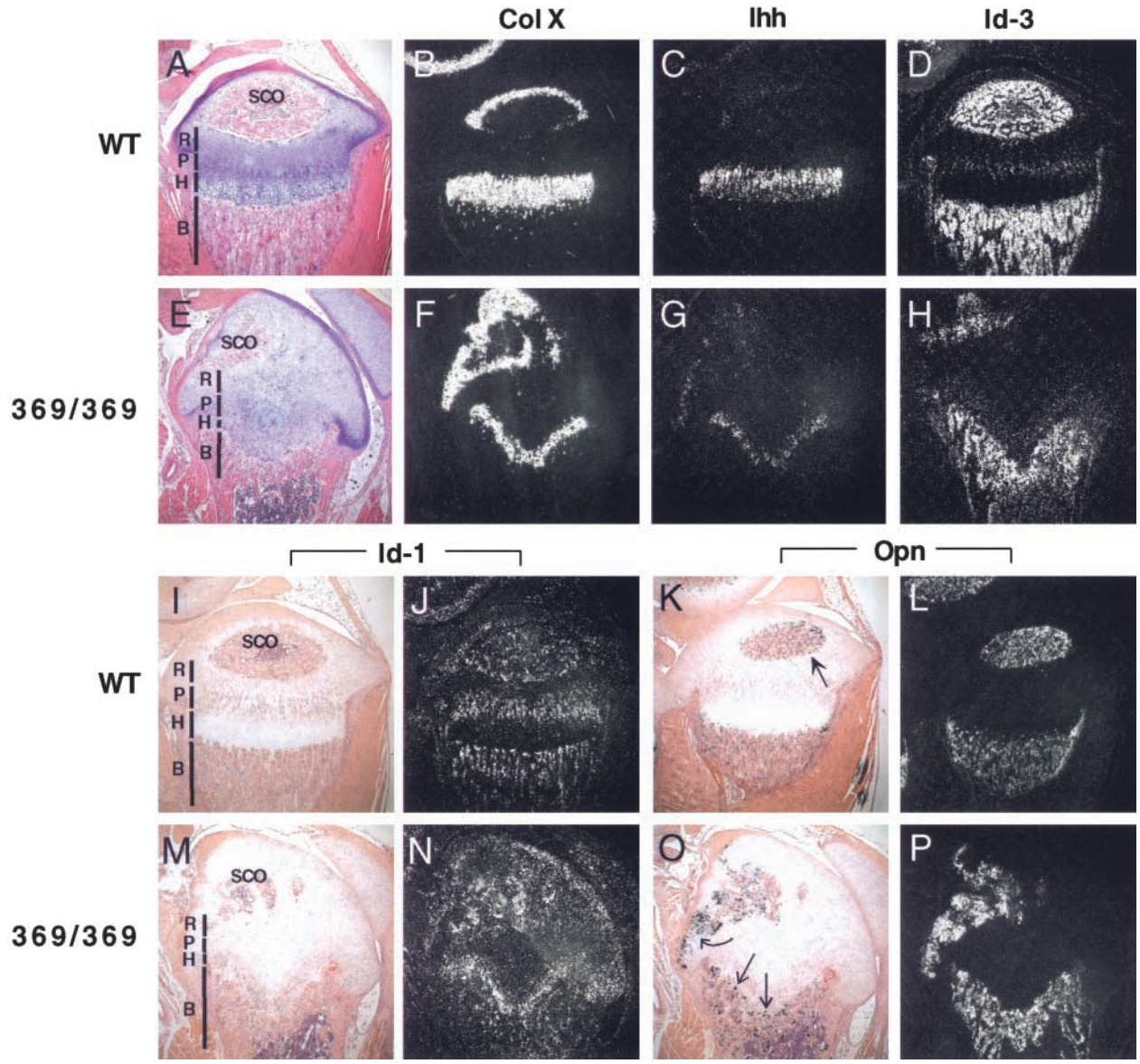

Figure 6. **In situ hybridization.** Tibia sections from P15 wild-type (WT; A–D and I–L) or FGFR3 mutant (369/369; E–H and M–P) mice were stained with Alcian blue, hemotoxylin, and eosin (A and E). R, P, and H depict the regions of the reserve, proliferative, and hypertrophic zones, respectively; B indicates trabecular bone and sco the secondary center of ossification. Sections were hybridized with ^{35}S-labeled RNA probes for collagen X (ColX; B and F), Indian hedgehog (Ihh; C and G), Id-3 (D and H), Id-1 (J and N), or osteopontin (Opn; L and P) and visualized using darkfield microscopy. After hybridization, sections in J, L, N, and P were stained with hemotoxylin to more precisely localize the regions expressing Id-1 and Opn (I, K, M, and O). Arrows indicate chondrocytes expressing OPN.

pertrophic chondrocytes, consistent with previous reports (Karsenty and Wagner, 2002). Expression of collagen X was, as expected, restricted to hypertrophic chondrocytes that could be observed near the trabecular bone and at the periphery of the secondary center of ossification. OPN expression was also detected in the hypertrophic cells, although its expression appeared stronger in and around the secondary centers of ossification and at the bone front, reflecting its additional expression by osteoblasts. The expression of Id1 and Id3 was particularly interesting because their mRNAs were detected only in proliferating chondrocytes and in newly formed bone (presumably by osteoblasts), but were completely absent in the resting and hypertrophic chondrocyte populations (Fig. 6).

Growth plates from the tibia of P15 mice homozygous for the activating FGFR3 mutation (369/369) exhibit a distorted V shape with a relative scarcity of hypertrophic chondrocytes at the bone front and poorly organized secondary centers of ossification (Chen et al., 1999). Ihh expression in these mutant growth plates was weak, as has been previously observed in similar mouse models of unregulated FGF signaling (Fig. 6; Karsenty and Wagner, 2002). Expression of OPN was strongly induced in the narrow band of hypertrophic chondrocytes as well as in osteoblasts in the mutant growth plate compared with its expression in the wild-type mouse. Id1 and Id3 expression were reduced in the proliferating chondrocytes of the mutant growth plate compared with that observed in the wild type, and most of their ex-

pression was detected only in osteoblasts of the trabecular bone. Collagen X appears weaker than that in the wild-type growth plate, although this decrease is likely due to the decrease in the number of hypertrophic cells expressing this marker, rather than, per se, a decrease in collagen X expression. Indeed, several cells expressing collagen X could be detected around the secondary centers of ossification in somewhat ectopic locations.

Together, these observations show that FGF causes changes in the expression pattern of several genes in RCS cells that are similar to those occurring during chondrocyte differentiation in vivo. Furthermore, the altered expression of these genes in the growth plates of mice harboring an activating mutation of FGFR3 mimics the changes observed in FGF-treated RCS cells, consistent with the notion that excessive FGF signaling may promote premature differentiation of chondrocytes in the mutant growth plates. However, the observation that collagen X expression is not induced by FGF signaling in either cultured or growth plate chondrocytes suggests that FGF does not promote all aspects of the differentiation program.

Discussion

Genetic studies of several forms of human dwarfism and mouse models of chondrodysplasic syndromes have established that FGF signaling plays a central role in normal bone growth by regulating both chondrocyte proliferation and differentiation in the epiphyseal growth plate. The developmental pathology caused by excessive FGF signaling as well as the unique, negative growth response of chondrocytes to FGF have prompted several studies to identify the intracellular effectors of FGF signaling in these cells. Although several individual molecules have been implicated in the FGF response, we sought to create a framework in which to extend these previous observations by documenting the global patterns of gene expression changes occurring over a 24-h period in a chondrocytic cell line in response to FGF, and by correlating these changes with alterations of the cell cycle. Our results, as discussed in the following paragraphs, are consistent with a model in which FGF signaling initiates an interlocking network of signaling and transcriptional events that act in a concerted and synergistic fashion to impact on chondrocyte proliferation and differentiation.

Multiple pathways contribute to growth arrest in response to FGF

The cell cycle is controlled by both positive and negative factors that ultimately determine the activity of the E2F family of transcription factors. Free E2Fs activate transcription of a host of target genes, many of which are critical to cell cycle progression, whereas E2Fs bound to hypophosphorylated Rb family members mediate transcriptional repression (Sherr and Roberts, 1999; Trimarchi and Lees, 2002). Because both p107 and p130 are essential for FGF-mediated growth arrest of chondrocytes (Laplantine et al., 2002), much of the focus of the present work was to determine the molecular events initiated by FGF that lead to p107 and p130 hypophosphorylation.

Initiation of growth arrest

FACScan™ analysis showed that RCS cells begin to arrest in the G1 phase by 6 h of FGF treatment (Fig. 1). In accordance with our previous works underscoring an important role for p107 and p130 proteins in this process (Laplantine et al., 2002), our gene expression data show that a subset of E2F target genes are rapidly down-regulated before growth arrest (Fig. 4). We further determined that p107 is the only Rb family member that is hypophosphorylated at very early times (Fig. 5), and thus the dephosphorylation of p107 and the repression of a subset of E2F target genes is likely to be the first key event leading to FGF-mediated growth arrest of RCS cells. It is unlikely that the dephosphorylation of p107 is due to changes in composition of the cyclin–Cdk complexes because we do not observe a down-regulation of gene expression for any of the components of the cyclin–Cdk machinery (cyclins B, D, and E, p55cdc, CDC5, CDC25B, CAK1, Cdk2; Table I), before growth arrest. In fact, the only change noted is the induction of cyclin D1 expression (Table I).

Our results also indicate that p107 hypophosphorylation does not result from the inhibition of cyclin E–Cdk activity. The microarray data show rapid induction of the CDKI p21 mRNA at 1 h and p16/Ink4a slightly later at 3 h (Table I). p21 binds both cylinE–Cdk2 and cyclin D–Cdk complexes, but only inhibits the kinase activity of the former (Sherr and Roberts 1999). In agreement with Aikawa et al. (2001), we have observed inhibition of cyclin E–Cdk2 activity as well as an increased association of p21 with the complex after 12 h of FGF treatment. However, we did not observe significant inhibition of cyclin E–Cdk2 or its association with p21 during the first 6 h of FGF treatment. Furthermore, the rapid dephosphorylation of p107 occurs even in the presence of actinomycin or cycloheximide, and thus ought not to rely on the synthesis of new products such as p21 (or p16). Although we did not directly assess cyclin D–Cdk function, our observation that the pRb and p130 proteins remain phosphorylated during the first 6 h of FGF treatment and that phosphorylation of these proteins by cyclin E complex is thought to be dependent on prior phosphorylation by cyclin D–Cdk (Harbour and Dean, 2000) are consistent with sustained cyclin D–Cdk activity during this period.

Together, these data illustrate a mechanism of p107-E2F–mediated transcriptional repression and growth arrest that directly result from FGF signaling. This interpretation is based on the observation that neither the dephosphorylation of p107 nor the repression of several of the E2F target genes requires new protein synthesis, and that these events occur independently of Cdk inhibition. As we have previously proposed (Laplantine et al., 2002), FGF signaling may activate a phosphatase or the association of a phosphatase with p107, a possibility that is currently under investigation. Clearly, the direct signaling mechanism for p107 dephosphorylation and the initiation of growth arrest elucidated here is distinct from that of growth arrest resulting from serum withdrawal and Cdk inhibition, and is a defining feature of the response of chondrocytes to FGF.

In addition to its role in binding E2Fs and repressing E2F target gene transcription, hypophosphorylated p107 has been shown to interact with, and negatively regulate the ac-

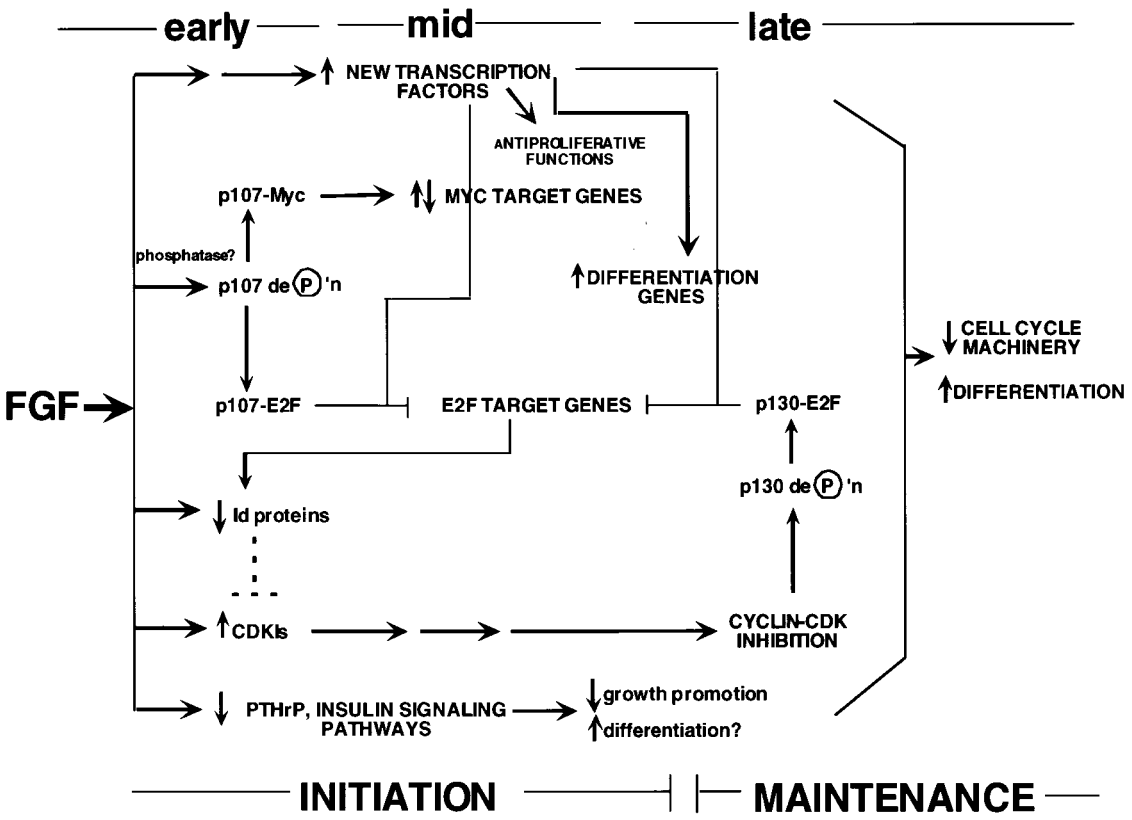

Figure 7. **Model depicting the multiple pathways induced in response to FGF treatment of RCS cells leading to cell cycle arrest and initiation of differentiation.** The initiation of growth arrest encompasses gene expression changes and signaling events observed during the early and mid-response stages. The major pathways serve to down-regulate growth-promoting functions and activate antiproliferative functions as discussed in the text. These events converge to establish the maintenance of growth arrest, which is characterized by cyclin E–Cdk inhibition, the hypophosphorylation of all three pRbs, further down-regulation of E2F target genes and genes encoding other cell cycle components, and the induction of genes associated with hypertrophic differentiation during the mid- and late response stages.

tivity of, several additional cellular factors including cyclin E–Cdk and c-Myc (Beijersbergen et al., 1994; Gu et al., 1994; Zhu et al., 1995). Because c-Myc activity is generally believed to regulate transcription of genes involved in growth control and is required for cell cycle progression (Grandori et al., 2000), interaction of hypophosphorylated p107 with c-Myc could also affect the expression of this additional set of target genes. We have noted changes in the expression of c-Myc target genes (Grandori and Eisenman, 1997), such as the down-regulation of Id2 and the induction of p21, that are consistent with the inhibition of myc activity (Table I and unpublished observations).

Maintenance of growth arrest

In contrast to the kinetics of p107 dephosphorylation, the appearance of underphosphorylated forms of pRb and p130 coincide with cyclin E–Cdk2 inhibition and depends on the activation of new gene transcription, presumably of a CDKI. Furthermore, Cdk inhibition and hypophosphorylated pRb and p130 proteins are observed after growth arrest had been initiated, presumably by p107, and therefore are unlikely to play a role in the early stages of this process. Although p107 plays a dominant role in growth inhibition of FGF-treated chondrocyte micromass or organ cultures, p130 is also required to elicit maximal levels of growth arrest (Laplantine et al., 2002). In addition, p107−/− mice exhibit a subtle bone phenotype compared with the severe chondrodysplasia manifest in p107−/−;p130−/− mice (Cobrinik et al., 1996). Thus, p130 may contribute unique functions not shared by p107 and/or may sustain the growth arrest at later stages in the FGF response.

Unlike the initiation of growth arrest, establishment of the maintenance phase relies on the earlier induction of new proteins such as the CDKIs. As a corollary to the CDKI-mediated Cdk inhibition that characterizes the maintenance phase, the early elimination of Id protein expression (Table I) is likely to be an additional essential aspect of the response of RCS cells to FGF. The Id proteins act as dominant-negative repressors of bHLH transcription factors and have been shown to antagonize the expression of both p21 and p16 (Yokota and Mori, 2002). Negative regulation of CDKI expression by Id proteins is further demonstrated in vivo by the observations that Id1−/−;Id3−/− neuroblasts exit prematurely from the cell cycle and show elevated levels of p16 and p27 (Lyden et al., 1999). In addition, Lasorella and colleagues (Lasorella et al., 2000) have demonstrated that Id2 can bind the hypophosphorylated form of all three pRbs, a property that appears to interfere with pRb–mediated cell cycle arrest. Thus, the immediate down-regulation of all three Id protein mRNAs after FGF treatment of RCS cells

would play a role in the induction of the p21 and p16 CDKIs, as well as in the repression of E2F target gene transcription by pRb–E2F complexes.

Additional pathways contributing to cell cycle arrest

Repression of E2F target genes were observed with very different kinetics, with some down-regulated within 1 h and others after 10 h of FGF treatment (Fig. 5), despite the fact that p107 dephosphorylation is so rapid. One possibility is that the p107–E2F4/5 complex does in fact mediate the repression of all these genes in response to FGF, but that a longer half-life of some of the mRNAs masks the transcriptional shutoff. However, an additional possibility is suggested by a recent report showing that TGF-β–mediated transcriptional repression of the c-Myc promoter, an E2F target gene, requires the collaboration of p107–E2F complexes with Smad proteins (Chen et al., 2002). Thus, although the p107–E2F complex mediates gene repression, it may require additional DNA-binding cofactors to do so. The observation that down-regulation of some of our E2F target genes requires new protein synthesis could reflect the induction of such cofactors that are not expressed in RCS cells in the absence of FGF signaling. An additional mechanism by which FGF promotes growth arrest may rely on the inhibition of the expression of growth-stimulating molecules such as PTHrP receptor and IRS-1.

Together, these observations show that FGF treatment leads to a cascade of interlocking events that converge to inhibit chondrocyte proliferation, as summarized in Fig. 7. A perspective that allows for the induction of multiple pathways, as opposed to a linear series of events, offers an explanation for the observations that mice containing gene knockout of individual components of these pathways, such as STAT1, p21, p107, or p130, do not exhibit severe skeletal phenotypes because one or more pathways may be able to compensate for the loss of another.

FGF signaling initiates chondrocyte differentiation

Chondrocyte proliferation and differentiation in developing long bones has been shown to be regulated by a number of signaling molecules, most notably Ihh, PTHrP, BMPs, IGFs, and FGFs. Although it is well established that Ihh and PTHrP function in a negative feedback loop to promote chondrocyte proliferation and restrain differentiation, (Olsen et al., 2000; Wagner and Karsenty, 2001; Karsenty and Wagner, 2002), the identification of positive differentiation signals has been more elusive. A number of reports (for review see Ornitz and Marie, 2002) have suggested that FGF signaling inhibits chondrocyte differentiation. However, this is seemingly at odds with the general notion that growth arrest is a prerequisite of, or coordinated with, terminal differentiation. Indeed, our microarray analysis demonstrates that FGF modulates the expression of a number of genes in RCS cells (Table II) in a manner that is consistent with the view that FGF promotes, rather than inhibits, many aspects of chondrocyte hypertrophic differentiation. This conclusion was supported by in situ analysis in which the modulation of expression of several of these genes was determined in epiphyseal growth plates from wild-type mice as well as mice harboring an activating FGFR3 mutation. In addition to the gene expression changes of many differentiation markers we have identified (Table II), it is worth mentioning that down-regulation of FGFR3 expression, another feature of chondrocyte differentiation, has been previously observed in FGF-treated RCS cells (Rozenblatt-Rosen et al., 2002).

A recent report by Minina and colleagues (Minina et al., 2002), in which interactions among the FGF-, Ihh/PTHrP-, and BMP-signaling pathways were assessed for their effects on organ cultures of growth plate chondrocytes, also presented compelling evidence that FGF promotes hypertrophic differentiation. Their model suggests that FGF promotes both the onset of differentiation, through the down-regulation of the Ihh/PTHrP pathway, and an acceleration of the differentiation process in an Ihh/PTHrP-independent manner. The down-regulation of PTHrP (and Ihh) gene expression that we observe is consistent with Minina's identification of the onset of hypertrophic differentiation (Minina et al., 2002). In addition, because we have used a tissue culture system in which chondrocytes are removed from the complex signaling environment of the growth plate, the majority of gene expression changes we observe should occur independently of the Ihh/PTHrP pathway, and may represent those contributing to the acceleration of differentiation observed by Minina et al. (2002). Together, these observations support the notion that FGF induces many aspects of chondrocyte differentiation and indicate that the phenotype of FGF-induced chondrodysplasias could result from both decreased proliferation and premature differentiation of growth plate chondrocytes. Indeed, another feature of FGF-induced chondrodysplasias, increased chondrocyte apoptosis, also supports this view because high levels of apoptosis are characteristic of terminally differentiated chondrocytes (Sahni et al., 2001).

In conclusion, our observations support the notion that FGF signaling plays two major, related roles in the epiphyseal growth plate by causing the growth arrest of proliferating chondrocytes, and subsequently, initiating a program of hypertrophic differentiation. Thus, FGF would serve as a coordinator that links these two processes and synchronizes the activities of the different chondrocyte populations. Obviously, these functions of FGF are also subject to control by other signaling pathways that would influence the onset of these events. Our observation that expression of collagen X is not induced also suggests that additional signals are required to achieve the full terminally differentiated state. Thus, by coordinating growth arrest and onset of the differentiation program, FGF initiates a switch in the state of proliferating chondrocytes toward hypertrophy.

Materials and methods

Cell culture

Monolayer cultures of the RCS and ROS 17/2.8 osteosarcoma cells were maintained in DME and 10% FCS. Where indicated, FGF1 and heparin were added to growing cultures to final concentrations of 5 ng/ml and 5 ug/ml, respectively. In experiments using 20 ug/ml cycloheximide or 1 ug/ml actinomycin D, the inhibitors were added 15 min before FGF/heparin addition. Cells were prepared and analyzed for FACScan™ analysis as described previously (Laplantine et al., 2002).

RNA isolation and microarray analysis

Total RNA was isolated using TRIzol® (GIBCO BRL). 3–4 independent samples were prepared from FGF-treated RCS cells at each time point. Du-

plicate RNA samples were prepared from control RCS cultures and FGF and control samples of ROS cells. Duplicate samples were also prepared from RCS cells treated with cycloheximide for 0, 1, 3, or 6 h in the presence or absence of FGF. Biotinylated cRNA was prepared according to the protocols as detailed (Affymetrix, Inc.) and submitted to the Columbia University Microarray Facility (New York, NY) for hybridization to the RGU34A rat genome array and scanning using the GeneArray® scanner (Affymetrix, Inc.).

Data analysis

Each U34A chip contains oligonucleotide sequences corresponding to 8,799 rat mRNAs or ESTs. Initial analysis was performed using Microarray Suite 5.0 software (Affymetrix, inc.). Metrics files were downloaded into GeneSpring® software (Silicon Genetics) for all further manipulations. Normalization across all of the chips was performed by using the 50th percentile of all measurements as a positive control. Each measurement for each sample was divided by this value. The lower 10th percentile was used as a test for correct background subtraction ("per chip normalization"). The median of each gene's expression value over all of the samples was used as a synthetic positive for each gene and divided into all measurements for that gene ("per gene normalization"). The values for multiple samples of each time point were averaged and used for all further analyses. Genes that did not exhibit a minimum raw signal of 100 in at least one sample in the time course were filtered out, eliminating most nonexpressed genes. Genes induced or down-regulated by at least threefold were identified by comparison of the expression level in each FGF-treated sample with that in the untreated (heparin only) control sample and combined using Venn Diagrams to yield the final lists of "all up-regulated" and "all down-regulated." These gene lists were each used to construct the dendrograms of Fig. 2 using Pearson correlation. Gene subgroups encompassed within nodes along the tree were selected and subject to the "major expression analysis" function of the GeneSpring® program to generate the expression patterns accompanying the dendrograms of Fig. 2. Genes within these subgroups were hand selected to create Table I. Fold change calculations shown in Table I used the time 0 (heparin only) value for each cell line as its baseline reference.

For E2F target gene analysis, rat homologues of the human E2F target genes reported by Ren et al. (2002) were identified using UniGene (National Center for Biotechnology Information, Bethesda, MD), and were subjected to k-means clustering (standard correlation) according to their expression patterns in FGF-treated RCS cells. Genes not expressed at any of the time points were excluded from the analysis.

Northern analysis

^{32}P-radio labeled probe for PC3 was prepared from the excised cDNA insert of pBabe PuroPC3 provided by F. Tirone (Instituto di Neurobiologia CNR, Rome, Italy). DNA fragments of OPN and OPG were generated using RT-PCR of RNA from FGF-treated RCS cells and primers as follows: OPN-F, 5′-GTGTCCTCTGAAGAAACGGA-3′; OPN-R, 5′-CTCGGCACTATCGATCGCAT-3′; OPG-F, 5′-CCTCCTGCTAATTCAGAAAG-3′; OPG-R, 5′-CTGATGGTCTTCCTCAGACT-3′.

The identity of the purified PCR products (570 bp, OPN; 600 bp, OPG) was verified by DNA sequencing and radiolabeled with random priming using the ReadyProbe kit (Amersham Biosciences) and α[^{32}P]dCTP. 10 μg of RNA from FGF-treated or control RCS cells were used for Northern analysis.

In vitro kinase assays

cyclin E–associated kinase activity was measured as described previously (Matsushime et al., 1994) In brief, precleared cell lysates were incubated with 2 μg anti-cyclin E antibody (sc-481, Santa Cruz Biotechnology, Inc.) for 2 h at 4°C. Protein A Sepharose beads were added and incubated for 1 h at 4°C. The immunoprecipitates were washed and one-third analyzed for kinase activity using 1 μg Histone H1 substrate and 10 μCi γ[^{32}P]ATP.

Western analysis

Cell lysates were prepared in RIPA buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 5 mM EDTA, 0.1% SDS, 1% nedeoxycholate, and 1% Triton X-100) and subjected to Western analysis as described previously (Laplantine et al., 2002) using antibodies against pRb (G3–245; BD Biosciences), p107 (C-18; Santa Cruz Biotechnology, Inc.), p130 (R27020; Transduction Laboratories), p21(C-19; Santa Cruz Biotechnology, Inc.), or Cdk2 (sc-163; Santa Cruz Biotechnology, Inc.).

In situ hybridization

Posterior limbs of P15 wild-type or 369/369 mice were fixed overnight in 4% PFA, embedded in paraffin, and sectioned at 7-μm intervals. In situ hybridization was performed as described previously (Wang et al., 1998) using antisense β[^{35}S]UTP-RNA probes. To prepare the probes, the OPN RT-PCR product described earlier in Materials and methods was cloned into the pGEM T-Easy vector (Promega). cDNAs of rat collagen X, Ihh, Id1 and Id3, provided by Drs. H. Kronenberg (Massachusetts General Hospital, Boston, MA), A. McMahon (Harvard University, Cambridge, MA), and R. Benezra (Memorial Sloan-Kettering Cancer Center, New York, NY), were each cloned into Bluescript. 20 ug of each plasmid DNA were linearized and transcribed in the presence of γ[^{35}S]UTP (PerkinElmer) using the appropriate polymerase (T7, T3, or SP6; Promega). After hybridization and washing, autoradiography was performed using Kodak NBT2 emulsion, and the slides were exposed for 1–2 wk. The slides were counterstained using hematoxylin. Hybridized probes were visualized using a dark field–equipped microscope. Contiguous sections of those analyzed by in situ hybridization were also triple stained with Alcian blue (Sigma-Aldrich), hematoxylin, and eosin.

Online supplemental material

Complete data sets for all of the microarray experiments described in this report are available at http://www.jcb.org/cgi/content/full/jcb.200302075/DC1.

We wish to thank V. Milikovic of the Columbia University Microarray Facility and U. Klein and M. Blumenberg for helping to set up the microarray experiments. We also thank F. Tirone, R. Benezra, H. Kronenberg, and A. McMahon for providing plasmids; F. Rossi and D. Cobrinik (Columbia University College of Physicians and Surgeons, New York, NY) and C.X. Deng (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD) for providing bone sections; T. Lufkin for advising on the in situ hybridizations; and A. Mansukhani for critical reading of the manuscript.

This work was supported by National Institutes of Health grant DE13745 from the National Institute of Dental and Craniofacial Research.

Submitted: 12 February 2003
Revised: 14 May 2003
Accepted: 14 May 2003

References

Aikawa, T., G.V. Segre, and K. Lee. 2001. Fibroblast growth factor inhibits chondrocytic growth through induction of p21 and subsequent inactivation of cyclin E-Cdk2. *J. Biol. Chem.* 276:29347–29352.

Beijersbergen, R.L., E.M. Hijmans, L. Zhu, and R. Bernards. 1994. Interaction of c-Myc with the pRb-related protein p107 results in inhibition of c-Myc-mediated transactivation. *EMBO J.* 13:4080–4086.

Brugarolas, J., C. Chandrasekaran, J.I. Gordon, D. Beach, T. Jacks, and G.J. Hannon. 1995. Radiation-induced cell cycle arrest compromised by p21 deficiency. *Nature.* 377:552–557.

Chen, C.R., Y. Kang, P.M. Siegel, and J. Massague. 2002. E2F4/5 and p107 as Smad cofactors linking the TGFbeta receptor to c-myc repression. *Cell.* 110: 19–32.

Chen, L., R. Adar, X. Yang, E.O. Monsonego, C. Li, P.V. Hauschka, A. Yayon, and C.X. Deng. 1999. Gly369Cys mutation in mouse FGFR3 causes achondroplasia by affecting both chondrogenesis and osteogenesis. *J. Clin. Invest.* 104:1517–1525.

Cobrinik, D., M.H. Lee, G. Hannon, G. Mulligan, R.T. Bronson, N. Dyson, E. Harlow, D. Beach, R.A. Weinberg, and T. Jacks. 1996. Shared role of the pRB-related p130 and p107 proteins in limb development. *Genes Dev.* 10: 1633–1644.

Deng, C., A. Wynshaw-Boris, F. Zhou, A. Kuo, and P. Leder. 1996. Fibroblast growth factor receptor 3 is a negative regulator of bone growth. *Cell.* 84: 911–921.

Durbin, J.E., R. Hackenmiller, M.C. Simon, and D.E. Levy. 1996. Targeted disruption of the mouse Stat1 gene results in compromised innate immunity to viral disease. *Cell.* 84:443–450.

Grandori, C., and R.N. Eisenman. 1997. Myc target genes. *Trends Biochem. Sci.* 22:177–181.

Grandori, C., S.M. Cowley, L.P. James, and R.N. Eisenman. 2000. The Myc/Max/Mad network and the transcriptional control of cell behavior. *Annu. Rev. Cell Dev. Biol.* 16:653–699.

Gu, W., K. Bhatia, I.T. Magrath, C.V. Dang, and R. Dalla-Favera. 1994. Binding and suppression of the Myc transcriptional activation domain by p107. *Science.* 264:251–254.

Harbour, J.W., and D.C. Dean. 2000. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes Dev.* 14:2393–2409.

Karsenty, G., and E.F. Wagner. 2002. Reaching a genetic and molecular understanding of skeletal development. *Dev. Cell.* 2:389–406.

Laplantine, E., F. Rossi, M. Sahni, C. Basilico, and D. Cobrinik. 2002. FGF signaling targets the pRb-related p107 and p130 proteins to induce chondrocyte growth arrest. *J. Cell Biol.* 158:741–750.

Lasorella, A., M. Noseda, M. Beyna, Y. Yokota, and A. Iavarone. 2000. Id2 is a retinoblastoma protein target and mediates signalling by Myc oncoproteins. *Nature.* 407:592–598.

Li, C., L. Chen, T. Iwata, M. Kitagawa, X.Y. Fu, and C.X. Deng. 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.

Lyden, D., A.Z. Young, D. Zagzag, W. Yan, W. Gerald, R. O'Reilly, B.L. Bader, R.O. Hynes, Y. Zhuang, K. Manova, and R. Benezra. 1999. Id1 and Id3 are required for neurogenesis, angiogenesis and vascularization of tumour xenografts. *Nature.* 401:670–677.

Mansukhani, A., P. Bellosta, M. Sahni, and C. Basilico. 2000. Signaling by fibroblast growth factors (FGF) and fibroblast growth factor receptor 2 (FGFR2)-activating mutations blocks mineralization and induces apoptosis in osteoblasts. *J. Cell Biol.* 149:1297–1308.

Matsushime, H., D.E. Quelle, S.A. Shurtleff, M. Shibuya, C.J. Sherr, and J.Y. Kato. 1994. D-type cyclin-dependent kinase activity in mammalian cells. *Mol. Cell. Biol.* 14:2066–2076.

Minina, E., C. Kreschel, M.C. Naski, D.M. Ornitz, and A. Vortkamp. 2002. Interaction of FGF, Ihh/Pthlh, and BMP signaling integrates chondrocyte proliferation and hypertrophic differentiation. *Dev. Cell.* 3:439–449.

Mukhopadhyay, K., V. Lefebvre, G. Zhou, S. Garofalo, J.H. Kimura, and B. de Crombrugghe. 1995. Use of a new rat chondrosarcoma cell line to delineate a 119-base pair chondrocyte-specific enhancer element and to define active promoter segments in the mouse pro-alpha 1(II) collagen gene. *J. Biol. Chem.* 270:27711–27719.

Norton, J.D., R.W. Deed, G. Craggs, and F. Sablitzky. 1998. Id helix-loop-helix proteins in cell growth and differentiation. *Trends Cell Biol.* 8:58–65.

Olsen, B.R., A.M. Reginato, and W. Wang. 2000. Bone development. *Annu. Rev. Cell Dev. Biol.* 16:191–220.

Ornitz, D.M., and P.J. Marie. 2002. FGF signaling pathways in endochondral and intramembranous bone development and human genetic disease. *Genes Dev.* 16:1446–1465.

Ren, B., H. Cam, Y. Takahashi, T. Volkert, J. Terragni, R.A. Young, and B.D. Dynlacht. 2002. E2F integrates cell cycle progression with DNA repair, replication, and G(2)/M checkpoints. *Genes Dev.* 16:245–256.

Rossi, F., H.E. MacLean, W. Yuan, R.O. Francis, E. Semenova, C.S. Lin, H.M. Kronenberg, and D. Cobrinik. 2002. p107 and p130 coordinately regulate proliferation, Cbfa1 expression, and hypertrophic differentiation during endochondral bone development. *Dev. Biol.* 247:271–285.

Rozenblatt-Rosen, O., E. Mosonego-Ornan, E. Sadot, L. Madar-Shapiro, Y. Sheinin, D. Ginsberg, and A. Yayon. 2002. Induction of chondrocyte growth arrest by FGF: transcriptional and cytoskeletal alterations. *J. Cell Sci.* 115: 553–562.

Sahni, M., D.C. Ambrosetti, A. Mansukhani, R. Gertner, D. Levy, and C. Basilico. 1999. FGF signaling inhibits chondrocyte proliferation and regulates bone development through the STAT-1 pathway. *Genes Dev.* 13:1361–1366.

Sahni, M., R. Raz, J.D. Coffin, D. Levy, and C. Basilico. 2001. STAT1 mediates the increased apoptosis and reduced chondrocyte proliferation in mice overexpressing FGF2. *Development.* 128:2119–2129.

Shen, M., E. Yoshida, W. Yan, T. Kawamoto, K. Suardita, Y. Koyano, K. Fujimoto, M. Noshiro, and Y. Kato. 2002. Basic helix-loop-helix protein DEC1 promotes chondrocyte differentiation at the early and terminal stages. *J. Biol. Chem.* 277:50112–50120.

Sherr, C.J., and J.M. Roberts. 1999. CDK inhibitors: positive and negative regulators of G1-phase progression. *Genes Dev.* 13:1501–1512.

Tirone, F. 2001. The gene PC3(TIS21/BTG2), prototype member of the PC3/BTG/TOB family: regulator in control of cell growth, differentiation, and DNA repair? *J. Cell. Physiol.* 187:155–165.

Trimarchi, J.M., and J.A. Lees. 2002. Sibling rivalry in the E2F family. *Nat. Rev. Mol. Cell Biol.* 3:11–20.

Vairapandi, M., A.G. Balliet, B. Hoffman, and D.A. Liebermann. 2002. GADD45b and GADD45g are cdc2/cyclinB1 kinase inhibitors with a role in S and G2/M cell cycle checkpoints induced by genotoxic stress. *J. Cell. Physiol.* 192:327–338.

Wagner, E.F., and G. Karsenty. 2001. Genetic control of skeletal development. *Curr. Opin. Genet. Dev.* 11:527–532.

Wang, Q., R.P. Green, G. Zhao, and D.M. Ornitz. 2001. Differential regulation of endochondral bone growth and joint development by FGFR1 and FGFR3 tyrosine kinase domains. *Development.* 128:3867–3876.

Wang, W., T. Van De Water, and T. Lufkin. 1998. Inner ear and maternal reproductive defects in mice lacking the Hmx3 homeobox gene. *Development.* 125:621–634.

Yan, G.Z., and E.B. Ziff. 1995. NGF regulates the PC12 cell cycle machinery through specific inhibition of the Cdk kinases and induction of cyclin D1. *J. Neurosci.* 15:6200–6212.

Yokota, Y., and S. Mori. 2002. Role of Id family proteins in growth control. *J. Cell. Physiol.* 190:21–28.

Zhan, Q., M.J. Antinore, X.W. Wang, F. Carrier, M.L. Smith, C.C. Harris, and A.J. Fornace, Jr. 1999. Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. *Oncogene.* 18: 2892–2900.

Zhu, L., E. Harlow, and B.D. Dynlacht. 1995. p107 uses a p21CIP1-related domain to bind cyclin/cdk2 and regulate interactions with E2F. *Genes Dev.* 9:1740–1752.