SUPPLEMENTAL INFORMATION

Gene expression profiling of PFR3^{K650E} expressing cells highlights constitutive activation of signaling pathways

Through multiple lines of research, knowledge regarding the signaling networks and signaling molecules activated by ligand stimulation of FGFRs has emerged (6, 39). To investigate signaling pathways activated by FGFR3 mutations through an analysis of altered expression of downstream transcriptional targets, we performed gene expression profiling of stably transfected inducible PC12 cells expressing a chimeric receptor (PFR3) consisting of the extracellular domain of human platlet derived growth factor receptor (PDGFR) and the transmembrane and intracellular domains of human FGFR3 (36) (described in main text).

Rat Affymetrix GeneChips were performed on induced PFR3^{K650E} verses induced PFR3 cells and comparison analyses were generated. To simplify discussion, genes which showed altered expression were placed in broad functional groups; major categories include metabolism [e.g. carbohydrate metabolism (galactosyltransferase, UDP galactose epimerase, GLUT1, aldehyde dehydrogenase), and oxygen metabolism (e.g., isocitrate dehydrogenase 1, ATP synthase, ATPase)], cell cycle regulation (e.g. cyclin D1, cyclin D2, p21$^{WAF1/CIP1}$), signaling [(e.g. FGFR1, TGFβ1, acetylcholine receptor, MAPKK, early growth response1 (egr1)], and cytoskeleton proteins (e.g. myosin heavy chain) (Table 1). Overall we found 65 genes with altered expression (i.e. 52 with increased expression and 13 with decreased expression). Furthermore overall gene expression differences (i.e. Fold Change) were small: 86% (45/52) of genes with increased expression were ≤ +3-fold and 96% (12/13) of genes with decreased expression were ≥-3-fold. These results suggest that in our PC12 model, PFR3^{K650E}, the mutant receptor causes modest alterations in transcription.

The expression of 4 genes, aldehyde dehydrogenase, cyclin D2, egr1, and p21$^{WAF1/CIP1}$ was evaluated by Northern blot and approximate fold change and direction of change for each was confirmed (data not shown and see below). One of the upregulated genes, p21$^{WAF1/CIP1}$, induced 1.7 fold, was confirmed by both Northern (1.4 fold) and Western blot analyses (Fig. 1). This gene was of particular interest as

$p21^{WAF1/CIP1}$ induction triggers cell growth arrest and is a downstream target of STAT1 and ERK1/2 signaling, implicated in FGFR3-mediated skeletal dysplasias. Indeed, expression changes of the majority of the altered genes are consistent with activation of ERK and/or STAT signaling, including genes such as egr1, $p21^{WAF1/CIP1}$, and MAP kinase phosphatase 1 (MKP-1). A number of genes are in common with those activated upon NGF treatment of PC12 cells (indicated by asterisk in Table 1) (40), associated with MAPK signaling. Cyclin D1, typically an ERK1/2 and STAT3 target gene, was found to be decreased in expression along with cyclin D2. Both of these proteins are upregulated in human cancers where proliferation is dysregulated (41); the observed downregulation is consistent with cell cycle arrest and ligand-independent differentiation observed in the K650E expressing cells. Finally, carbohydrate and lipid metabolism can also be regulated through the action of ERK1/2 and STAT signaling (42, 43). Several induced genes are also in common with those identified by gene expression profiling in the presence of FGF in rat chondrosarcoma (RCS) cells, a chondrocyte cell line that primarily expresses the FGFR3 form of the FGF receptor types (44). Therefore, gene expression profiling of ligand-independent signaling is most consistent with an enhancement of normal FGFR3 signaling that can occur in the presence of ligand, as opposed to the activation of novel signaling pathways.

Materials and Methods

RNA sample preparation and microarrays

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

For all the GeneChips, the average fluorescence intensity was scaled to 500 arbitrary units so that chips could be directly compared. All 9 possible pairwise comparisons were made between induced PFR3 and $PFR3^{K650E}$ lines using the Affymetrix Microarray Suite v5.0 (Santa Clara, CA). Genes were considered significant if 50% of the pairwise comparisons were called changed (i.e. Difference Call was increased, moderately increased, decreased or moderately decreased). MAS v5.0 Signal Log Ratio values were converted to fold change values and averaged across all pairwise comparisons. Probe pairs were selected that had an average fold change of at least +/- 1.5.

Table 1.		
GenBank #	**Gene Product**	**FC**
	Stress Response	
S63521	glucose-regulated protein GRP78	4.3
S81478	* 3CH134/CL100 PTPase, MKP-1	1.5
	Cytoskeletal	
M12098	embryonic sarcomeric myosin heavy chain	5.3
X03369	beta-tubulin T beta15	1.9
AA818677	neurofilament, heavy polypeptide	3.1
J02962	lectin, galactose binding, soluble 3	2.1
AA800948	similar to Tubulin alpha-4 chain	2.4
L13039	calpactin I heavy chain	1.7
	Growth Factors/Signaling	
L04485	MAP kinase kinase	1.5
L31621	cholinergic receptor, nicotinic, alpha polypeptide 3	1.5
J05231	cholinergic receptor, nicotinic, alpha polypeptide 5	1.9
D37880	TYRO3 protein tyrosine kinase 3	2.3
M18416, AF023087, U75397	* early growth response 1	3.1
M74223	VGF nerve growth factor inducible	1.5
S54008	Fibroblast growth factor receptor 1	2.9
AI071299, AI172476	TGFB inducible early growth response	1.8
X52498	* transforming growth factor, beta 1	3.4
AI007824	similar to Guanine nucleotide exchange factor MSS4	1.6
X06916	* S100 calcium-binding protein A4	1.6
D00575	glycoprotein hormones, alpha subunit	1.6
AA859878, AI639318	* ret proto-oncogene	1.8
X77235	ADP-ribosylation-like 4	1.5
M83679	RAB15	2.4
AA799551	similar to Rab family small GTPase Rah	-3.3
U27767	regulator of G-protein signaling 4	-1.5
AI175776	retroviral-like c-Ha-ras proto-oncogene	-2.5
	Cell cycle/Replication	
U24174	p21WAF1/CIP1	1.7
D14014, X75207	cyclin D1	-1.6
AA899106	cyclin D2	-2.9
	Transcription/Translation	
AF009330	enhancer-of-split and hairy-related protein 2 (SHARP-2	1.5
U83883	p105 coactivator	1.5
AF104399	melanocyte-specific gene 1 protein	1.9
AI178828	eukaryotic translation initiation factor 4E binding protei	1.8
	Metabolism	
AF048687	UDP-Gal:betaGlcNAc beta, polypeptide 6	2.3
D26073	PRPP synthetase-associated protein	6.3
D37920	squalene epoxidase	1.7
J02585	stearoyl-Coenzyme A desaturase 1	1.9
M29249	hydroxy-3-methylglutaryl coenzyme A reductase	1.8
M58364	GTP cyclohydrolase 1	1.8
X53949	galactose-4-epimerase, UDP	1.6
AA875269	stearoyl-Coenzyme A desaturase 2	1.6
AA892314	isocitrate dehydrogenase 1	1.7
U81186	17 beta-hydroxysteroid dehydrogenase type 3	1.5
AA799778	ATP synthase, H+ transporting, F0 complex, subunit b	1.9
Y12635	ATPase, H+ transporting, beta 56/58 kDa	1.5
S68135	GLUT1=glucose transporter 1	1.5
AF065438	peptidylprolyl isomerase C-associated protein	-1.5
AF097723	plasma glutamate carboxypeptidase	-1.8
AF001898	aldehyde dehydrogenase family 1, member A1	-2.9
	Protein Turnover	
D17296	ubiquitin C	-1.9
	Miscellaneous and Unidentified	
X05472	Rat 2.4 kb repeat DNA right terminal region	3.8
X76489	CD9 antigen	2.1
X62875	high mobility group AT-hook 1	2.2
AA892511	similar to Tescalcin	1.5
E12625	novel protein which is expressed with nerve injury	1.8
U03416	neuronal olfactomedin-related ER localized protein	1.6
AA800315	similar to PxF protein	6.0
H31479	similar to RA175	1.6
AA893032	EST	1.6
AA893454	EST	1.7
AA874848	EST	1.7
AA874803	EST	-1.9
AA874803	EST	-1.7
AA874873	EST	-2.0
AI639312	EST	-1.9
* denote NGF-inducible genes		

Table S1. Genes with altered expression in induced PFR3^{K650E} (mutant) cells relative to induced PFR3 (wildtype) cells. Affymetrix U34A GeneChips were probed with cRNA synthesized from RNA from mutant or wildtype cells induced for 24 hr as decribed in the Materials and Methods. Genes listed in the table were called changed in at least 50% of the pairwise comparisons using MAS v5.0 software. FC refers to the average fold change values determined in MAS v5.0 for all possible pairwise comparsions. For cases where mulitple probe pair sets were identified for the same gene, the corresponding Genbank Accession Numbers are given and the fold change is the average for all probe sets. Only genes with average fold changes less than -1.5 or greater than +1.5 were considered. * denote NGF-inducible genes.