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Analysis of the Epidermal Growth Factor Receptor Specific Transcriptome: Effect of Receptor Expression Level and an Activating Mutation

Mikkel W. Pedersen,[1] Nina Pedersen,[1] Lars Damstrup,[1] Mette Villingshøj,[1] Søren U. Sønder,[2] Klaus Rieneck,[2] Lone F. Bovin,[2] Mogens Spang-Thomsen,[3] and Hans S. Poulsen[1]*

[1]Department of Radiation Biology, The Finsen Center, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark
[2]Institute for Inflammation Research, The Finsen Center, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark
[3]Institute of Molecular Pathology, University of Copenhagen, DK-2100 Copenhagen, Denmark

Abstract Overexpression or expression of activating mutations of the epidermal growth factor receptor (EGFR) is common in cancer and correlates with neoplastic progression. The present study employed Affymetrix® oligonucleotide arrays to profile genes induced by ligand-activated EGFR with the receptor either moderately expressed or overexpressed at an in-itself transforming level. These changes were compared to those induced by the naturally occurring constitutively active variant EGFRvIII. This study provides novel insight on the activities and mechanisms of EGFRvIII and EGFR mediated transformation, as genes encoding proteins with functions in promoting cell proliferation, invasion, anti-apoptosis, and angiogenesis featured prominently in the EGFRvIII- and EGFR-expressing cells. Surprisingly, it was found that ligand-activated EGFR induced the expression of a large group of genes known to be inducible by interferons. Expression of this module was absent in the EGFRvIII-expressing cell line and the parental cell line. Treatment with the specific EGFR inhibitor AG1478 indicated that the regulations were primary, receptor-mediated events. Furthermore, activation of this module correlated with activation of STAT1 and STAT3. The results thus demonstrate that ligand-activated EGFR at different expression levels results in different kinetics of signaling and induction of gene expression. In addition, the constitutively active variant EGFRvIII seems to activate only a subset of signal pathways and induce a subset of genes as compared to the ligand-activated EGFR. J. Cell. Biochem. 96: 412–427, 2005.

Key words: EGFR; EGFRvIII; interferon; signaling; STAT; affymetrix; expression profiling

The epidermal growth factor receptor (EGFR) is a transmembrane receptor tyrosine kinase involved in regulation of cell proliferation, growth, survival, and motility [Ullrich et al., 1984; Di Fiore et al., 1987; Wells et al., 1998]. By now, the role of EGFR in the pathogenesis of a broad range of human cancers is well established and the mechanisms of how EGFR can become oncogenic are many. These include: autocrine growth factor loops, overexpression, and deletions or mutations, which render the receptor independent of ligand and constitutively active [Wong et al., 1992; Damstrup et al., 1999; Peghini et al., 2002]. Recent evidence suggests that genetic alterations in the EGFR gene may be as important as overexpression with respect to the oncogenic potential and to correlate with poor prognosis [Schwechheimer et al., 1995; Sugawa et al., 1998; Ge et al., 2002; Pandita et al., 2004].

The most common genetic alteration is the class III mutant EGFR (EGFRvIII, de2–7 EGFR, Δ2–7 EGFR). This aberrant receptor arises from an in-frame deletion of exons 2–7, giving rise to a mature mRNA lacking 801 nucleotides [Sugawa et al., 1990; Yamazaki et al., 1990;

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*Correspondence to: H. S. Poulsen, Department of Radiation Biology, The Finsen Center, Section 6321, Copenhagen University Hospital, Blegdamsvej 9, DK-2100 Copenhagen, Denmark. E-mail: skovgaard@rh.dk

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Wong et al., 1992]. The deletion eliminates 267 amino acids (aa 6–273) from the extracellular domain resulting in a constitutively active receptor with a distorted ligand binding area and a unique glycine residue at the fusion junction [Sugawa et al., 1990]. A large number of studies now support the role of EGFRvIII in the genesis and progression of human cancers, as EGFRvIII is able to transform fibroblasts in vitro and enhance the tumorigenicity of cancer cells both in vitro and in vivo [Moscatello et al., 1996; Pedersen et al., 2001a; Damstrup et al., 2002].

The global transcriptional profile induced by EGFR and EGFRvIII, however, remains largely undefined, and virtually nothing is known of how overexpression of EGFR and expression of activating mutations of EGFR influence signaling and global gene transcription.

In the present study, we have utilized oligonucleotide arrays (Affymetrix®, Santa Clara, CA) to characterize changes in the transcriptome induced by ligand-activated EGFR or the constitutively active variant EGFRvIII in mammalian cell lines. The unique steps used here include comparison of the transcriptional changes induced by transforming and non-transforming levels of EGFR in the presence or absence of EGF as well as by EGFRvIII. Furthermore, the contributions of EGFRvIII/EGFR tyrosine kinases to transcriptional changes were investigated using the small molecular weight inhibitor AG1478, a specific EGFR tyrosine kinase inhibitor. The experiments demonstrate that ligand-activated EGFR expressed at different levels results in different kinetics of signaling and induction of gene expression. In addition, the constitutively active variant EGFRvIII seems to activate only a subset of signal pathways and induce a subset of genes compared to that of ligand-activated EGFR.

MATERIALS AND METHODS

Materials

Recombinant human EGF and AG1478 were purchased from Calbiochem (Germany). EGFR, phospho-EGFR, STAT1, STAT3, phospho-STAT1, phospho-STAT3, AKT, phospho-AKT, ERK-1/2, and phospho-ERK-1/2 antibodies were from Cell Signaling Technology (Germany). Antibodies to Tubulin, FRA-1, and EGR-1 were from Santa Cruz Biotechnology, Inc. (CA). Antibody to muPAR was a kind gift from Dr. Gunilla Høyer-Hansen, Finsen Laboratory, Copenhagen, Denmark. HRP-conjugated secondary antibodies were purchased from DAKO (Denmark).

Cell Lines and Tumors in Nude Mice

Six cell lines were included in this work. The cell lines NR6, NR6M, and NR6W have been described previously [Batra et al., 1995] and were kindly provided by Dr. Darell Bigner, Duke University, NC. Briefly, NR6M and NR6W were generated by transfection of NR6, a variant of the Swiss 3T3 murine fibroblast cell line that lacks endogenous EGFR, with cDNAs encoding full-length human EGFR (NR6W) or the type III mutant EGFR (NR6M). The NR6wtEGFR cell line, which expresses a lower number of receptors as compared to NR6W, has also been described previously and is a generous gift from Dr. Allan Wells, Department of Pathology, University of Pittsburgh [Wells et al., 1990; Chen et al., 1994]. A431 cells were obtained from American Type Culture Collection. U87MG and U87MG-ΔEGFR cells were obtained from Dr. Webster Cavenee (San Diego, CA). All cells were maintained in DMEM (Life Technologies, Inc., Scotland) supplemented with 10% fetal calf serum (FCS), 50 U/ml penicillin, and 50 μg/ml streptomycin. For the GeneChip and Northern blot analyses, cells were seeded in DMEM with 10% FCS, allowed to grow for 24 h until 80% confluent, washed once in phosphate buffered saline, and serum starved in DMEM supplemented with 0.5% fetal calf serum for 24 h. Serum-starved cells were treated with the solute control DMSO for 3 h (MOCK), MOCK and 10 nM EGF for 1 h, or 10 μM AG1478 (in DMSO) (EGFR/EGFRvIII tyrosine kinase inhibitor) for 3 h, and 10 nM EGF for 1 h. NR6M cells released from AG1478 inhibition were serum starved for 24 h and treated with AG1478 for 24 h prior to release.

For Western blot analyses, cells were grown as above, serum starved, and subsequently treated with inhibitor for 3 h and/or EGF for 24 h.

Tumor xenografts were generated by bilateral inoculation of 10^7 cells in each flank of 6-week-old female BALB/cA-nu nude mice (Taconic, Ry Denmark). Six mice were used for each cell line. The mice were observed daily, and when visible tumors appeared, the tumors were measured in two perpendicular dimensions (d1 and d2), three times a week using a

sliding gauge. Tumor areas ($A = d1 \times d2$) and volumes ($V = 0.35 \times A^{3/2}$) were calculated and used to construct mean growth curves of tumor volume, and according to a transformed Gompertz function. Construction of mean growth curves and calculation of the tumor volume doubling times (TD) were performed as previously described [Rygaard and Spang-Thomsen, 1997].

Soft Agar Assay for Anchorage-Independent Growth

Exponentially growing cells (1×10^5) were suspended in 3 ml 0.5% (w/v) NuSieve low melting agar (FMC, Rockland, ME) dissolved in DMEM + 10% FCS and covered with 0.5% agar dissolved in DMEM + 10% FCS in six-well plates. Cultures in triplicate for each condition were replenished with fresh medium once a week. After 3 weeks, the plates were stained with crystal violet and colonies >50 cells were counted visually.

RNA Extraction and Hybridization

The preparation of biotin cRNA was prepared essentially as described in the Affymetrix Expression Analysis Technical Manual. Briefly, 10 μg of RNA was used as template to generate double-stranded cDNA using a T7-$(dT)_{24}$ primer (Genset, France) using SuperScript RnaseH$^-$ Reverse Transcriptase and subsequent second-strand synthesis (Invitrogen). The cDNA was transcribed into biotin-labeled cRNA using the BioArray, High Yield RNA transcript labeling kit (Enzo Diagnostics, Farmingdale, NY). Fragmentation, hybridization, and scanning were according to the Affymetrix® protocol using the Affymetrix® murine oligonucleotide MG-U74Av2 arrays (Affymetrix, Inc., Santa Clara, CA), which contain 12,000 probe sets corresponding to 6,000 ESTs and 6,000 annotated genes. To minimize false positives, all samples were analyzed in duplicate with RNA harvested from cells from independent experiments.

Data Filtering and Analysis

The 22 CEL files generated by the Affymetrix Microarray Suite (MAS) version 5.0 were converted into DCP files using dCHIP (www.dCHIP.org), as described previously by Li and Wong [2001]. The DCP files were normalized, and raw gene expression data generated using the PM—only model contained in the dCHIP software. In the current experiment, duplicate samples were found to have small variability.

Genes that were differentially expressed were identified by filtering levels of gene-specific signal intensity for statistically significant differences, when grouped based on conditions using an ANOVA test, P-value cutoff 0.05, and an absolute correlation. These genes were then further filtered to deselect genes having no expression value of 50 or more in any of the samples. Seven hundred eighty seven genes passed this filtering.

An unsupervised hierarchical cluster analysis was performed on the 787 genes with dCHIP [Li and Wong, 2001] using Pearson's correlation distance metric and centroid linkage.

Northern Blot Analyses

Five micrograms of total RNA, isolated from serum-starved cells or tumor tissue using Trizol reagent, was size fractionated on a 1% agarose-formaldehyde gel in MOPS buffer and transferred to a magma nylon membrane (MSI, MA). The 18S and 28S ribosomal bands, having a ratio of approximately 1:2, verified the integrity of the RNA. The cDNA probes for Decorin (Dcn, GenBank Acc. X53929), Thrombospondin-2 (Thbs2, L07803), Fos like antigen-1 (Fosl1, AF017128), Proliferin (prlf, K02245), Early growth response-1 (egr1, M28845), Urokinase plasminogen activator receptor (muPAR, X62700), Epiregulin (Ereg, D30782), Glyceraldehyde 3-phosphate dehydrogenase (Gapdh, NM_002046), Suppressor of cytokine signaling 3 (Socs3, U88328), Interferon-inducible GTPase (IIGP, AJ007971), Interferon-regulatory factor 1 (irf1, M21065), and Interferon gamma inducible protein (ifi47, M63630) were generated by reverse transcription-polymerase chain reaction (RT-PCR) with primer sets generating cDNA products of approximately 500 bp.

All primers were from DNA Technology (Denmark). The probes were labeled with [(-^{32}P]dCTP using the multiprime DNA labeling system RPN1601 (Amersham Pharmacia Biotech, UK). Membranes were hybridized with the probes in ExpressHyb™ hybridization solution (Clontech, CA) according to the manufacturer's directions. The membranes were exposed to Kodak X-ray film with an intensifying screen at −80°C for autoradiography.

Immunoblot Analyses

For determination of phosphorylated proteins, cells were lysed directly in 1× NuPAGE sample buffer (Invitrogen, Denmark), immediately

separated on precast NuPAGE SDS–PAGE gels (Invitrogen), and electroblotted onto nitrocellulose membranes. After transfer, blocking was done by incubation in 5% non-fat milk. Primary antibody staining was done by incubation overnight at 40°C, and secondary antibody staining was for 1 h at room temperature. The chemiluminescence detection method was used for all Western blot experiments. For verifications of GeneChip results, 5 μg whole cell lysate was resolved by SDS–PAGE and electroblotted onto nitrocellulose membranes. Blotting and antibody incubations were performed as above.

RESULTS

EGFRvIII and Overexpression of EGFR Induces Transformation of Fibroblasts

To evaluate the effect of EGFR and EGFRvIII expression on the NR6 phenotype, the anchorage-independent growth properties of EGFR and EGFRvIII expressing NR6 cells were investigated. The parental cell line was not able to form colonies in soft-agar. The EGFRvIII-expressing cell line (NR6M) formed large colonies in the presence and absence of ligand (Fig. 1a). The low-EGFR-expressing cell line

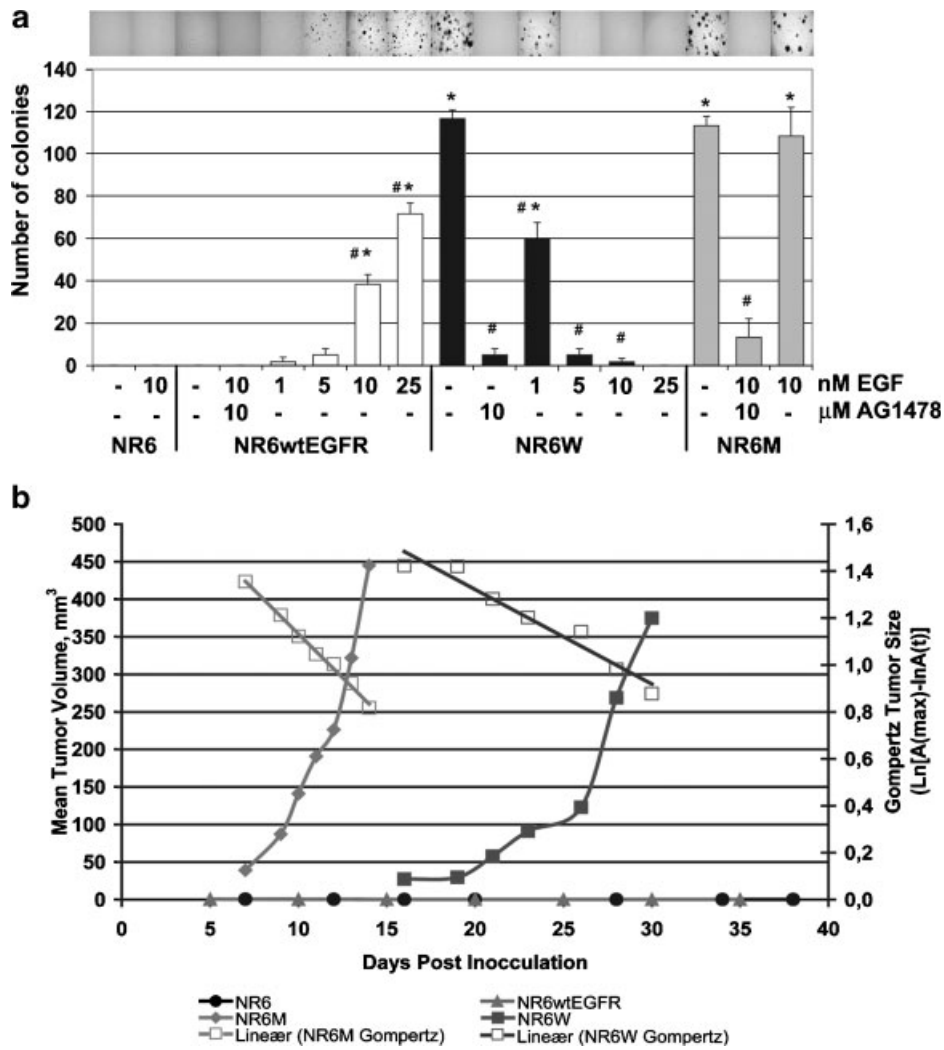


Fig. 1. Transformation of NR6 cells by EGFRvIII and EGFR. **a**: Colony formation of NR6, NR6wtEGFR, NR6W, and NR6M cells in soft agar in the presence and absence of AG1478, and EGF. Plates were stained after 3 weeks and colonies larger than 50 cells counted (the mean from three experiments was $^{*}P < 0.01$ compared to the control cell line, NR6 and $^{\#}P < 0.05$ compared to the untreated cell line using Student's paired *t*-test. **b**: Only the NR6W and NR6M cell lines grew as xenografts. The mean transformed Gompertz growth curve depicts the growth as a straight line when the tumor size ln[lnA(max)–lnA(t)] is plotted against time (right y-axis). Mean tumor volume (left y-axis) was calculated from mean tumor area. A(max) is the theoretical maximal tumor area, A(t) is tumor area at time t, and the lines are the best fit regression lines of the mean transformed Gompertz functions.

(NR6wtEGFR) was dependent on ligand stimulation for development of colonies. In contrast, the high-EGFR-expressing cell line (NR6W) formed numerous colonies in the absence of ligand, an ability that was inhibited by EGF stimulation. The ability of all three cell lines to form colonies was strongly inhibited by AG1478.

The capacity to form tumors in athymic nude mice is the hallmark of neoplastic transformation; therefore, the ability of the different cell lines to form tumors in nude mice was investigated. Within 6 days after s.c. injection of NR6M cells, all mice (6/6) developed tumors, which progressed rapidly (Fig. 1b). Likewise, NR6W cells were able to form tumors (6/6) in athymic mice, but only after a latency period of 15 days. In addition to a faster tumor establishment, NR6M tumors had a calculated doubling time of 2.9 days, which was approximately half that of NR6W tumors (5.3 days). Tumor growth was not detected in any of the mice injected with either NR6wtEGFR cells or NR6 cells in the experimental period of 40 days.

Genome-Wide Expression Profiling Reveals Distinct and Overlapping Gene Expression Mediated by High and Low EGFR Expression and Expression of EGFRvIII

To identify genes that could explain some of the variations in cell behavior described in Figure 1, genome-wide expression analyses were performed using the Affymetrix Murine GeneChip oligonucleotide array Mu74Av2, which allows the simultaneous study of more than 6,000 murine genes and 6,000 ESTs. The mRNA levels were measured in the NR6, NR6wtEGFR, NR6W, and NR6M cell lines with or without addition of 10 nM EGF for 1 h. Furthermore, AG1478 was used to identify genes whose expression was dependent on EGFR/EGFRvIII tyrosine kinase activity. To visualize the gene expression data, hierarchical clustering was performed using the 787 genes that satisfied the stringent filtering criteria. Eight major clusters of genes C1–C8 were identified on the dendrogram (Fig. 2). Zoomed

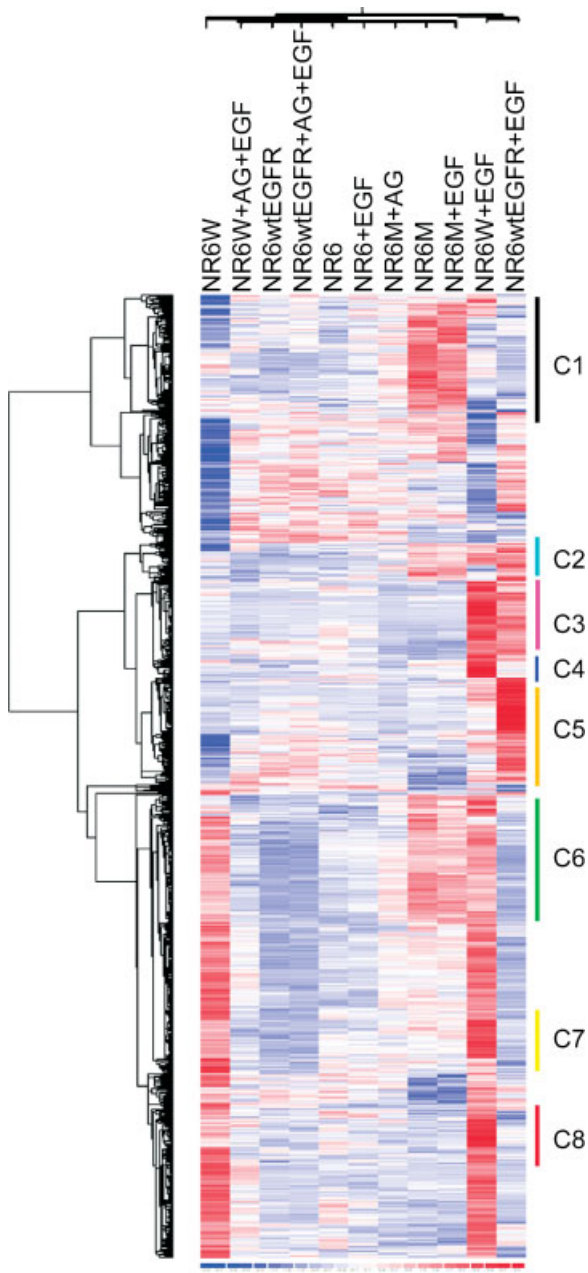


Fig. 2. Gene expression profiles of the 11 samples analyzed. The 787 differentially expressed genes were identified by ANOVA analysis using the following criteria: $P < 0.05$ and signal >50 in one of the samples. To visualize the data, genes and samples were grouped using hierarchical clustering and graphically represented in dCHIP. Red colors represent genes with high expression whereas blue colors denote low expression. Prominent clusters are highlighted with colored bars to the right of the figure. *Cluster C1* holds genes induced solely by EGFRvIII, while *Cluster C2* is composed of genes that are stimulated by ligand-activated EGFR and, although more weakly, constitutively active EGFRvIII. *Cluster C3* includes genes induced by ligand-activated EGFR at both expression levels but not by EGFRvIII. *Clusters C4* and *C5* contain genes induced exclusively by EGF stimulation of the high- and low-EGFR-expressing cell line, respectively. *C6* is composed of genes induced by EGFR in the high-EGFR-expressing cell line and by EGFRvIII but not by EGFR in the low-expressing cell line. Two smaller *Clusters C7* and *C8* contain genes stimulated by EGF in the high-EGFR-expressing cell line and inhibited by AG1478, but with a relatively high expression in the unstimulated cells. AG: AG1478.

images of the kinetically distinguishable clusters C2, C3, C5, and C6, which depict differences and similarities between ligand-activated EGFR expressed at two different levels and EGFRvIII, are shown in Figure 3. The remaining clusters can be found in the supplementary material. Of the eight clusters, the genes in clusters C2–5 are strictly EGF-stimulated genes, as their expression is absent in the unstimulated EGFR expressing cell lines.

The genes in *Cluster C2* (Fig. 3) are induced by ligand stimulation of EGFR as well as by EGFRvIII, and are dependent on the tyrosine kinase activity of the receptors, as AG1478 decreases their expression. Ligand-activated EGFR in the low-EGFR-expressing cell line is more competent in inducing expression of these genes than activated receptors in the high expressing cell line, whereas EGFRvIII is the least competent. This cluster includes genes previously shown to be induced by serum growth factors including Fos like antigen 1 (*Fosl1, Fra-1*), muPA receptor 1 (*Plaur*), Epiregulin (*Ereg*), Kruppel-like factor 5 (*Klf5*), Ankyrin repeat domain 1 (*Ankrd1*), and Myelocytomatosis oncogene (*Myc*) [Fambrough et al., 1999; Iyer et al., 1999; Sweeney et al., 2001].

Cluster C3 (Fig. 3) holds genes that are induced by ligand-activated EGFR but not by EGFRvIII and also dependent on the tyrosine kinase activity of the receptor. Ligand-activated EGFR in the high EGFR expressing cell line is the most competent inducer of the genes in this cluster. Like *Cluster C2*, *Cluster C3* contains many immediate early genes induced by serum growth factors: Jun B oncogene (*JunB*), cysteine rich protein 61 (*Cyr61*), Serum inducible kinase (*Snk*), Serum/glucocorticoid regulated kinase (*Sgk*), and Dual specificity kinase 1 (*Dusp1*).

The genes in *Cluster 4* (Supplementary material) are specifically induced by EGF stimulation of the high EGFR expressing cell line and include: FBJ osteosarcoma oncogene (*Fos*), Mitogen activated protein kinase kinase 3 (*Mkk3*), and Myeloid differentiation primary response gene 88 (*Mydd88*).

Like *Cluster C3*, *Cluster C5* contains genes that are induced by ligand-activated EGFR and are dependent on the tyrosine kinase activity of the receptors. Ligand-activated EGFR in the low EGFR expressing cell line is the most competent inducer of these genes. Once more, many of the genes in *Cluster C5* have previously been shown to be induced by serum growth factors: Jun B oncogene (*JunB*), Heparin binding EGF-like growth factor (*Dtr, HB-EGF*), Plasminogen activator urokinase (*Plau*), FBJ osteosarcoma oncogene B (*FosB*), Dual-specificity phosphatase 2 (*Dusp2*), Early growth response 1 (*Egr1*), and Cysteine rich protein 61 (*Cyr61*).

In general, many of the genes in *Cluster C2*, *C3*, *C4*, and *C5* have previously been correlated with proliferation, transformation, motility, and/or other malignant behavior of cells [Charles et al., 1993; Kaufmann and Thiel, 2001; Kjoller and Hall, 2001; Mazar, 2001; Normanno et al., 2001; Tsai et al., 2001; Carles-Kinch et al., 2002; Pelengaris et al., 2002]. It is interesting to note that at the time point studied, the expression of the genes in *Clusters C4, C7*, and *C8* seem to be induced more readily by ligand-activated EGFR in the high expressing cell line, whereas the genes in *Cluster C5* are more readily induced by ligand-activated EGFR in the low expressing cell line. EGFRvIII seems to be the weakest inducer of these genes.

One kinetically distinguishable cluster contains genes that are induced by both high level EGFR and EGFRvIII (Fig. 3, *Cluster C6*). The expression of the genes in this cluster does not seem to be induced by EGF in the timeframe studied (1 h) but are presumably secondary response genes with slower kinetics. This is supported by the observation that the high EGFR expressing cell line (NR6W), like NR6M, has a low level of constitutively active receptors and by the fact that a 3 h inhibition with AG1478 decreases the expression of the genes in these clusters [Pedersen et al., 2004]. A number of genes involved in control of cell growth and proliferation are found in this cluster including Cyclin D1 (*Ccnd1*), Ras p21 protein activator 1 (*Rasa1*), Neuroblastoma ras oncogene (*Nras*), Sprouty protein with an EVH-1 domain (*Spred2*), and Nucleoporin 88 (*Nup88*). This cluster was also enriched in genes (19 of 78) associated with DNA replication, translation, and protein synthesis, which make sense as mitogenic stimulation of resting cells leads to both a general increase in net protein synthesis and a specific increase in the synthesis of replication-promoting proteins.

Cluster C1 (Supplementary material) holds the genes with a high expression in NR6M cells only and whose expression is inhibited by AG1478 in this cell line. Fifty percent of these are EST's with unknown function and many

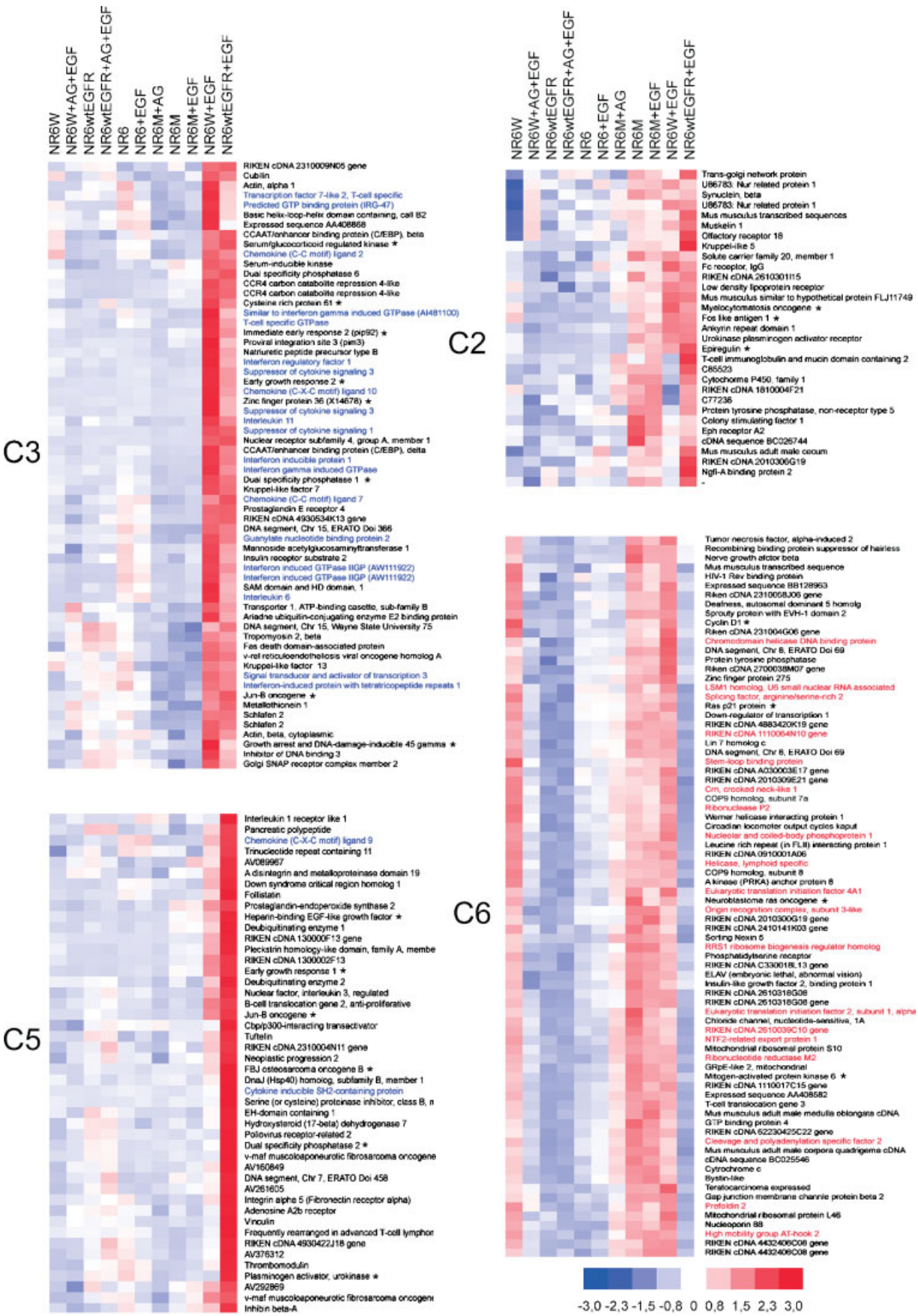

NR6W
NR6W+AG+EGF
NR6wtEGFR
NR6wtEGFR+AG+EGF
NR6
NR6+EGF
NR6M+AG
NR6M
NR6M+EGF
NR6W+EGF
NR6wtEGFR+EGF
C3
RIKEN cDNA 2310009N05 gene
Cubilin
Actin, alpha 1
Transcription factor 7-like 2, T-cell specific
Predicted GTP binding protein (IRG-47)
Basic helix-loop-helix domain containing, cell B2
Expressed sequence AA408868
CCAAT/enhancer binding protein (C/EBP), beta
Serum/glucocorticoid regulated kinase ★
Chemokine (C-C motif) ligand 2
Serum-inducible kinase
Dual specificity phosphatase 6
CCR4 carbon catabolite repression 4-like
CCR4 carbon catabolite repression 4-like
Cysteine rich protein 61 ★
Similar to interferon gamma induced GTPase (AI481100)
T-cell specific GTPase
Immediate early response 2 (pip92) ★
Proviral integration site 3 (pim3)
Natriuretic peptide precursor type B
Interferon regulatory factor 1
Suppressor of cytokine signaling 3
Early growth response 2 ★
Chemokine (C-X-C motif) ligand 10
Zinc finger protein 36 (X14678) ★
Suppressor of cytokine signaling 3
Interleukin 11
Suppressor of cytokine signaling 1
Nuclear receptor subfamily 4, group A, member 1
CCAAT/enhancer binding protein (C/EBP), delta
Interferon inducible protein 1
Interferon gamma induced GTPase
Dual specificity phosphatase 1 ★
Kruppel-like factor 7
Chemokine (C-C motif) ligand 7
Prostaglandin E receptor 4
RIKEN cDNA 4930534K13 gene
DNA segment, Chr 15, ERATO Doi 366
Guanylate nucleotide binding protein 2
Mannoside acetylglucosaminyltransferase 1
Insulin receptor substrate 2
Interferon induced GTPase IIGP (AW111922)
Interferon induced GTPase IIGP (AW111922)
SAM domain and HD domain, 1
Interleukin 6
Transporter 1, ATP-binding casette, sub-family B
Ariadne ubiquitin-conjugating enzyme E2 binding protein
DNA segment, Chr 15, Wayne State University 75
Tropomyosin 2, beta
Fas death domain-associated protein
v-rel reticuloendotheliosis viral oncogene homolog A
Kruppel-like factor 13
Signal transducer and activator of transcription 3
Interferon-induced protein with tetratricopeptide repeats 1
Jun-B oncogene ★
Metallothionein 1
Schlafen 2
Schlafen 2
Actin, beta, cytoplasmic
Growth arrest and DNA-damage-inducible 45 gamma ★
Inhibitor of DNA binding 3
Golgi SNAP receptor complex member 2
C2
Trans-golgi network protein
U86783: Nur related protein 1
Synuclein, beta
U86783: Nur related protein 1
Mus musculus transcribed sequences
Muskelin 1
Olfactory receptor 18
Kruppel-like 5
Solute carrier family 20, member 1
Fc receptor, IgG
RIKEN cDNA 2610301I15
Low density lipoprotein receptor
Mus musculus similar to hypothetical protein FLJ11749
Myelocytomatosis oncogene ★
Fos like antigen 1 ★
Ankyrin repeat domain 1
Urokinase plasminogen activator receptor
Epiregulin ★
T-cell immunoglobulin and mucin domain containing 2
C85523
Cytochrome P450, family 1
RIKEN cDNA 1810004F21
C77236
Protein tyrosine phosphatase, non-receptor type 5
Colony stimulating factor 1
Eph receptor A2
cDNA sequence BC026744
Mus musculus adult male cecum
RIKEN cDNA 2010306G19
Ngfi-A binding protein 2
-
C5
Interleukin 1 receptor like 1
Pancreatic polypeptide
Chemokine (C-X-C motif) ligand 9
Trinucleotide repeat containing 11
AV089967
A disintegrin and metalloproteinase domain 19
Down syndrome critical region homolog 1
Follistatin
Prostaglandin-endoperoxide synthase 2
Heparin-binding EGF-like growth factor ★
Deubiquitinating enzyme 1
RIKEN cDNA 1300000F13 gene
Pleckstrin homology-like domain, family A, membe
RIKEN cDNA 1300002F13
Early growth response 1 ★
Deubiquitinating enzyme 2
Nuclear factor, interleukin 3, regulated
B-cell translocation gene 2, anti-proliferative
Jun-B oncogene ★
Cbp/p300-interacting transactivator
Tuftelin
RIKEN cDNA 2310004N11 gene
Neoplastic progression 2
FBJ osteosarcoma oncogene B ★
DnaJ (Hsp40) homolog, subfamily B, member 1
Cytokine inducible SH2-containing protein
Serine (or cysteine) proteinase inhibitor, class B, n
EH-domain containing 1
Hydroxysteroid (17-beta) dehydrogenase 7
Poliovirus receptor-related 2
Dual specificity phosphatase 2 ★
v-maf musculoaponeurotic fibrosarcoma oncogene
AV160849
DNA segment, Chr 7, ERATO Doi 458
AV261605
Integrin alpha 5 (Fibronectin receptor alpha)
Adenosine A2b receptor
Vinculin
Frequently rearranged in advanced T-cell lymphor
RIKEN cDNA 4930422J18 gene
AV376312
Thrombomodulin
Plasminogen activator, urokinase ★
AV292869
v-maf musculoaponeurotic fibrosarcoma oncogen
Inhibin beta-A
C6
Tumor necrosis factor, alpha-induced 2
Recombining binding protein suppressor of hairless
Nerve growth factor beta
Mus musculus transcribed sequence
HIV-1 Rev binding protein
Expressed sequence BB128963
Riken cDNA 2310058J06 gene
Deafness, autosomal dominant 5 homolg
Sprouty protein with EVH-1 domain 2
Cyclin D1 ★
Riken cDNA 2310046G06 gene
Chromodomain helicase DNA binding protein
DNA segment, Chr 8, ERATO Doi 69
Protein tyrosine phosphatase
Riken cDNA 2700038M07 gene
Zinc finger protein 275
LSM1 homolog, U6 small nuclear RNA associated
Splicing factor, arginine/serine-rich 2
Ras p21 protein ★
Down-regulator of transcription 1
RIKEN cDNA 4883420K19 gene
RIKEN cDNA 1110054N10 gene
Lin 7 homolog c
DNA segment, Chr 8, ERATO Doi 69
Stem-loop binding protein
RIKEN cDNA A030003E17 gene
RIKEN cDNA 2010309E21 gene
Crn, crooked neck-like 1
COP9 homolog, subunit 7a
Ribonuclease P2
Werner helicase interacting protein 1
Circadian locomoter output cycles kaput
Nucleolar and coiled-body phosphoprotein 1
Leucine rich repeat (in FLII) interacting protein 1
RIKEN cDNA 0910001A06
Helicase, lymphoid specific
COP9 homolog, subunit 8
A kinase (PRKA) anchor protein 8
Eukaryotic translation initiation factor 4A1
Neuroblastoma ras oncogene ★
Origin recognition complex, subunit 3-like
RIKEN cDNA 2010300G19 gene
RIKEN cDNA 2410141K03 gene
Sorting Nexin 5
RRS1 ribosome biogenesis regulator homolog
Phosphatidylserine receptor
RIKEN cDNA C330018L13 gene
ELAV (embryonic lethal, abnormal vision)
Insulin-like growth factor 2, binding protein 1
RIKEN cDNA 2610318G08
RIKEN cDNA 2610318G08 gene
Eukaryotic translation initiation factor 2, subunit 1, alpha
Chloride channel, nucleotide-sensitive, 1A
RIKEN cDNA 2610039C10 gene
NTF2-related export protein 1
Mitochondrial ribosomal protein S10
Ribonucleotide reductase M2
GRpE-like 2, mitochondrial
Mitogen-activated protein kinase 6 ★
RIKEN cDNA 1110017C15 gene
Expressed sequence AA408582
T-cell translocation gene 3
Mus musculus adult male medulla oblongata cDNA
GTP binding protein 4
RIKEN cDNA 6220425C22 gene
Cleavage and polyadenylation specific factor 2
Mus musculus adult male corpora quadrigemina cDNA
cDNA sequence BC025546
Cytochrome c
Bystin-like
Teratocarcinoma expressed
Gap junction membrane channle protein beta 2
Prefoldin 2
Mitochondrial ribosomal protein L46
Nucleoporin 88
High mobility group AT-hook 2
RIKEN cDNA 4432406C08 gene
RIKEN cDNA 4432406C08 gene
-3,0 -2,3 -1,5 -0,8 0 0,8 1,5 2,3 3,0

Fig. 3.

of the remaining only scarcely characterized. Again, many of these genes are presumably slow response genes upregulated by the long-term activity of EGFRvIII.

In summary, the clustering analysis indicates that ligand-activated EGFR and EGFRvIII activates overlapping, but also distinct sets of genes in murine fibroblasts. It is also evident from the cluster analysis that the EGFRvIII and the high EGFR expressing cell lines have increased expression of numerous genes in the absence of ligand stimulation, which presumably is due to constitutive receptor activity in these cell lines. These genes, many with unknown function, are most likely involved in the development and maintenance of the transformed phenotype.

Verification of Selected mRNA and Protein Levels

To validate the Affymetrix oligonucleotide array results, the expression of a subset of genes with diverse expression profiles: Epiregulin *(Ereg)*, Early growth response 1 (*Egr1)*, mUPA receptor 1 *(Plaur1)*, Thrombospondin-2 *(Thrbs2)*, Proliferin *(Plf)*, Decorin *(Dcn)*, and Fos like antigen 1 *(Fsl1)*, were independently tested by Northern blot analyses (Fig. 4a). The relative levels of mRNAs calculated from the Northern blot analysis, when compared to the corresponding ratio determined in the Affymetrix analysis, produced remarkable concordance (data not shown).

Western blot analysis was performed to examine whether the observed changes in mRNA levels of EGR-1, FRA-1 (Fsl1), and mUPAR were reflected in their protein levels (Fig. 4b). As for the Northern blot analysis, we found a good correlation between gene expression data and the actual protein level for these three genes.

A Group of Genes Normally Associated With Stimulation by Interferon Is Induced by Ligand-Activated EGFR but not EGFRvIII

Intriguingly, many genes in *Cluster C3*, and some in *Clusters C5*, have mainly been associated with interferon stimulation, particularly interferon γ. These include genes encoding suppressor of cytokine signaling 1 and 3 (SOCS1 and SOCS3), interferon gamma regulatory factor 1 (IRF1), interferon inducible GTPase (IIGP, Iigp-pending), and interferon regulated gene 47 (IFI47) (See Fig. 5a,b for complete list and expression levels). Expression of the interferon module was completely absent in the EGFRvIII-expressing cell line. Their reduced expression upon treatment with AG1478 indicated that the regulation was a primary, receptor-mediated event. The expression levels of *Socs3*, *Irf1*, *IIGP*, and *Ifi47* were verified by Northern blot analyses (Fig. 5c). Time profiles show that the expression of all four genes is more rapid and more transient in the low EGFR expressing cell line than in the high expresser. To investigate if the lack of gene activation by EGFRvIII was a consequence of its constitutive activity, we simulated activation of EGFRvIII by releasing it from AG1478 inhibition (Fig. 5c). However, also under these experimental conditions, EGFRvIII was unable to induce expression of these genes.

Activation of the Signal Transducers and Activators of Transcription by Ligand-Activated EGFR but not by EGFRvIII

Since interferon-γ induced changes in gene expression are mediated mainly through the signal transducers and activators of transcription (STATs) factors, the levels of STAT1 and STAT3 activation (as measured by level of phosphorylation) were investigated downstream of ligand-activated EGFR and EGFRvIII. STAT1 and STAT3 were found to be phosphorylated at tyrosine residue 705 and 701 respectively by ligand-activated EGFR at both expression levels, whereas EGFRvIII was unable to activate these STATs (Fig. 6a). However, only ligand-activated EGFR in the low EGFR expressing cell line was able to induce phosphorylation of STAT3 on serine residue 727. The phosphorylations were directly receptor mediated as they were inhibited by addition of AG1478 (Fig. 6a).

Phosphorylations of STAT1 and STAT3 by EGF stimulation was verified in another EGFR expressing cell line A431, and in addition, it was found that EGFRvIII was unable to induce phosphorylation of STAT1 and 3 in the glioblastoma cell line U87MG (Fig. 6b).

Fig. 3. Close-up of gene *Clusters C2, C3, C5,* and *C6*. Genes in blue are interferon-regulated genes. Genes in red encode genes with products involved in DNA replication, translation, and protein synthesis. Genes marked with an asterisk indicate previously reported EGFR regulated gene.

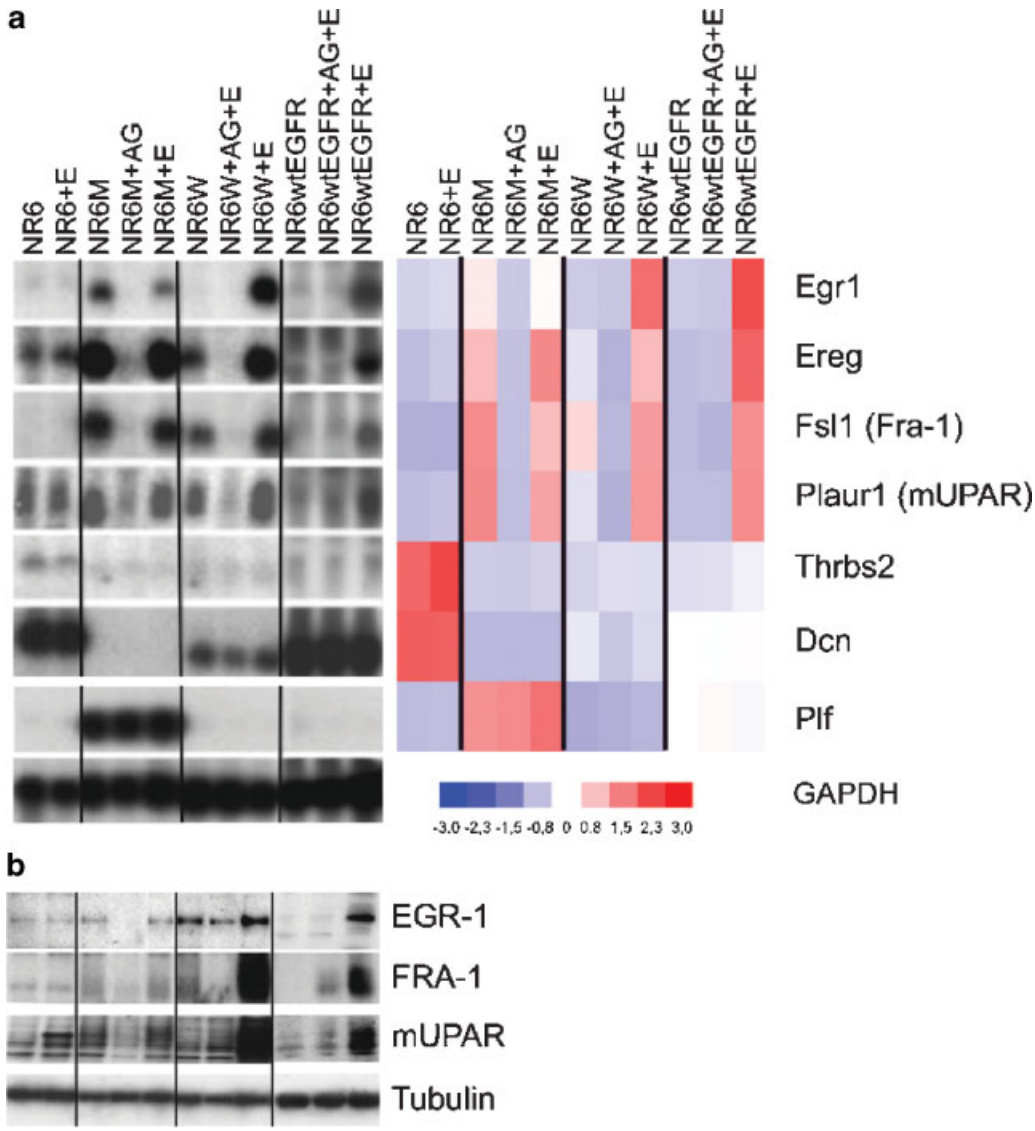


Fig. 4. **a**: Northern blot analyses of seven selected mRNA transcripts: Early growth response 1 (*Egr-1*), thrombospondin 2 (*Thrbs2*), epiregulin (*Ereg*), mouse urokinase plasminogen activator receptor (*muPAR*), Fos like antigen 1 (*Fsl1*), decorin (*Dcn*), and proliferin (*Plf*) confirm GeneChip probe array results. The level of the *GAPDH* transcript was included as a control of loading. Genechip results are shown to the right for comparison. **b**: Western blot analysis of FRA-1, EGR-1 and mUPAR levels, tubulin was included as control for loading. E:EGF, AG:AG1478. Experiments were repeated at least three times and representative blots are shown.

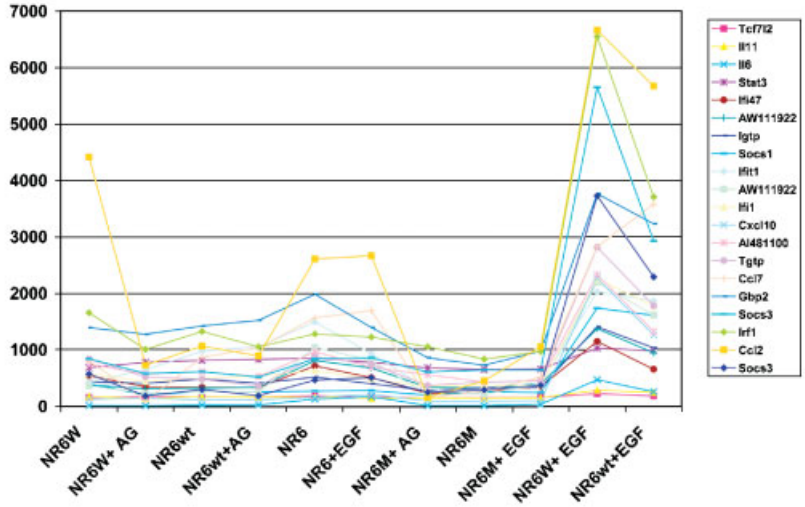
STAT Signaling Is Amplified in the EGFR Overexpressing Cell Line

To further characterize the activation of STAT1 and 3 by ligand-activated EGFR, a time profile study was performed (Fig. 7a), which demonstrated that activated EGFR in the low EGFR expressing cell line induces a more rapid activation of STAT1 and 3 that peaked after 10 and 60 min, respectively, compared to a slow but persistent activation in the high EGFR expressing cell line. In fact, the phosphorylation of STAT3 on tyrosine residue 705 does not seem to reach a maximum in the high EGFR expressing cell line in the time frame studied. EGF mediated STAT3 phosphorylation on serine residue 727 is only seen in the low EGFR expressing cell line. It was also investigated whether the lack of activation of STAT1 and STAT3 by EGFRvIII was due to its constitutive nature and thus its state of equilibrium. Again, releasing EGFRvIII from inhibition by AG1478

a

Probe set	Description	Gene Symbol	NR6W	NR6W+ AG	NR6wt	NR6wt+ AG	NR6	NR6+E GF	NR6M+ AG	NR6M	NR6M+ EGF	NR6W+ EGF	NR6wt+ EGF
101186_at	Transcription factor 7-like 2 T-cell specific	Tcf7l2	156	168	171	193	154	161	146	155	159	224	184
92296_at	Interleukin 11	Il11	172	141	176	162	164	143	149	148	155	287	248
102218_at	Interleukin 6	Il6	13	11	22	26	126	172	12	13	37	479	260
99099_at	Signal transducer and activator of transcription 3	Stat3	688	780	816	832	854	782	680	652	663	1028	985
104759_at	Interferon gamma inducible protein	Ifi47	543	345	337	335	718	507	229	254	349	1148	657
103963_1_at	Interferon induced GTPase IIGP	AW111922	378	311	326	335	812	680	337	326	400	1373	947
160833_at	Interferon gamma induced GTPase	Igtp	438	405	486	408	519	401	282	330	334	1408	1034
92832_at	Suppressor of cytokine signaling 1	Socs1	400	201	266	257	271	280	199	261	242	1739	1011
100981_at	Interferon-induced protein 1	Ifit1	428	660	976	1057	1483	934	309	211	405	2927	1867
96764_at	Interferon induced GTPase IIGP	AW111922	365	228	268	259	1044	811	206	223	372	2197	1614
97409_at	Interferon inducible protein 1	Ifi1	578	441	498	538	831	486	384	379	368	2256	1776
93858_at	Chemokine (C-X-C motif) ligand 10	Cxcl10	124	148	114	117	183	215	92	94	100	2283	1259
98410_at	Similar to interferon induced GTPase	AI481100	752	569	814	527	965	691	561	431	457	2327	1323
102908_at	T-cell specific GTPase	Tgtp	837	521	482	360	875	749	388	433	446	2816	1779
94761_at	Chemokine (C-C motif) ligand 7	Ccl7	809	96	866	1038	1566	1697	153	271	520	2830	3590
104597_at	Interferon inducible	Gbp2	1393	1276	1421	1521	1984	1395	856	730	977	3765	3231
92232_at	Suppressor of cytokine signaling 3	Socs3	843	581	615	517	844	858	609	644	645	5652	2931
102401_at	Interferon regulatory factor 1	Irf1	1655	1008	1326	1054	1281	1225	1054	837	970	6549	3707
102736_at	Chemokine (C-C motif) ligand 2	Ccl2	4414	730	1066	891	2609	2667	159	461	1056	8661	5672
162206_f_at	Suppressor of cytokine signaling 3	Socs3	572	184	360	188	486	599	256	294	370	3729	2290

b



c

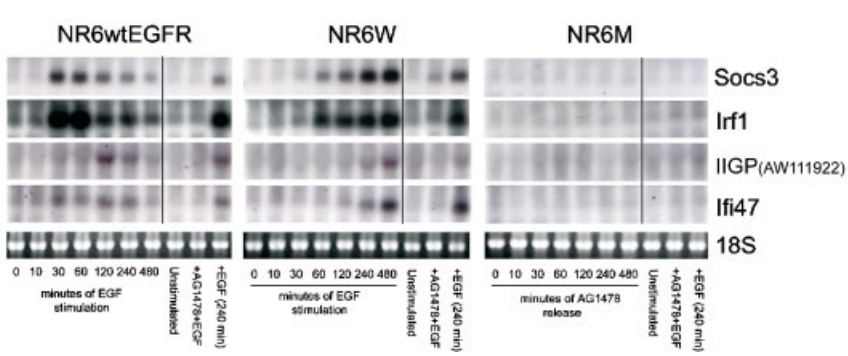


Fig. 5. **a**: List of genes in the INF module induced primarily by ligand-activated EGFR. For each gene, the average expression level (Affymetrix signal) in the samples, the probeset, the gene symbol, and a description is given (genes are found in *Cluster C3*). **b**: Detailed expression profiles. **c**: Time profiles of mRNA levels of four selected genes in the interferon module as found using Northern blot analysis: *Socs3*, *Irf1*, *IIGP*, and *Ifi47* upon EGF stimulation or release from 24 h of AG1478 inhibition (NR6M). The effect of receptor inhibition on the expression of these genes by AG1478 is likewise shown. The 18S ribosomal bands confirm equal loading. Experiments were repeated at least three times and representative blots are shown.

simulated activation, but no phosphorylation of either STAT1 or STAT3 could be detected (data not shown).

The effect of EGFR activation level on the activation of STAT1 (Tyr701) and STAT3 (Tyr705) was investigated by stimulating the low and high EGFR expressing cell lines with increasing concentrations of EGF (Fig. 7b). STAT3 phosphorylation level continues to increase with increasing levels of EGFR phosphorylation, whereas the STAT1 phosphorylation level reaches a maximum after stimulation with 10 nM EGF. This is in contrast to ERK activation, which reaches maximum phosphorylation level in both cell lines after stimulation with 0.1 nM EGF, although a weaker activation was observed in the high EGFR expressing cell line.

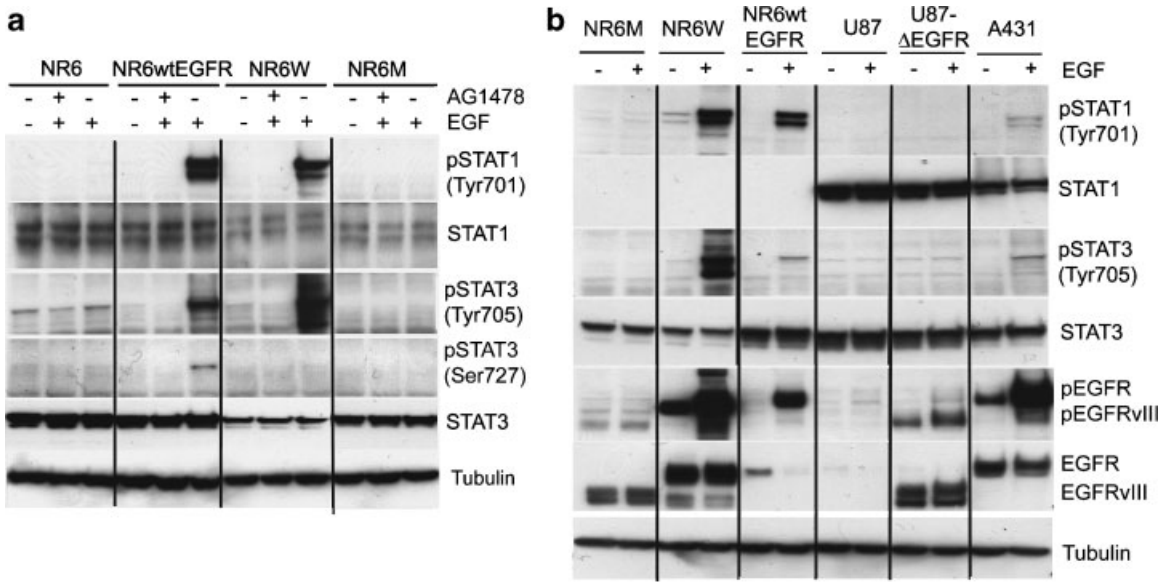


Fig. 6. **a**: Western blot analysis showing STAT1 and STAT3 phosphorylations in NR6wtEGFR and NR6W cells. Whole cell lysates of NR6, NR6wtEGFR, NR6W, and NR6M cells treated with the indicated inhibitor and/or EGF were subjected to Western blot analysis using antibodies directed against phosphorylated STAT1/3 (pSTAT) or total STAT1/STAT3. Equal amount of protein loading was confirmed by detection of tubulin. **b**: Western blot analysis of STAT1 and STAT3 phosphorylations in the cell lines NR6M, NR6W, NR6wtEGFR, U87MG, U87MG-ΔEGFR, and A431. Whole cell lysates of cells treated with EGF were subjected to Western blot analysis using antibodies directed against phosphorylated STAT1/3 (pSTAT) or total STAT1/STAT3. Total phosphorylation levels as well as the total levels of EGFR and EGFRvIII in the samples are shown for comparison. Equal amount of protein loading was confirmed by detection of tubulin. Experiments were repeated at least three times and representative immunoblots are shown.

DISCUSSION

Overexpression and expression of mutated versions of EGFR are frequently associated with the development of a number of human tumors including those of the lung, ovary, breast, prostate, and in particular those of the brain [Wong et al., 1992; Garcia et al., 1993; Moscatello et al., 1995; Olapade-Olaopa et al., 2000]. In this study, we have used a model system consisting of four cell lines either not expressing EGFR (NR6), expressing EGFR at a low level (NR6wtEGFR), expressing EGFR at a high transforming level (NR6W), or expressing EGFRvIII (NR6M) to address these important questions. While the parental cell line NR6 is incapable of forming colonies in soft agar and tumors in nude mice, the transformed cell lines NR6W and NR6M readily form colonies in soft-agar and tumors in nude mice (Fig. 1a,b). The NR6wtEGFR cell line is dependent on high concentrations of EGF for colony formation, but is unable to form tumors in nude mice at the selected concentration of cells. Recently, we have shown that NR6wtEGFR, NR6W, and NR6M have increased motility compared to NR6, and that this increase in motility is dependent on activated EGFR and EGFRvIII [Pedersen et al., 2004]. Thus, this study provides not only a direct evaluation of the EGFR and EGFRvIII regulated transcriptional programs but also a molecular characterization of phenotypic changes induced in the NR6 cell line upon expression and activation of EGFR and EGFRvIII. We here show that the transformed cell lines NR6W and NR6M have constitutive expression of a large number of genes regulated by EGFR, as compared to NR6 and NR6wtEGFR. Expression of these genes is dependent on receptor tyrosine kinase activity as illustrated by their decreased expression in response to the inhibitor AG1478. This is meaningful, since EGFR in NR6W and EGFRvIII are constitutively phosphorylated and able to activate downstream signaling in the absence of ligand stimulation [Moscatello et al., 1996; Huang et al., 1997; Pedersen et al., 2004]. Many of these genes have not previously been described as being EGFR inducible and are presumably secondary or tertiary response genes dependent on transcription of immediate early genes [Fambrough et al., 1999; Iyer et al., 1999].

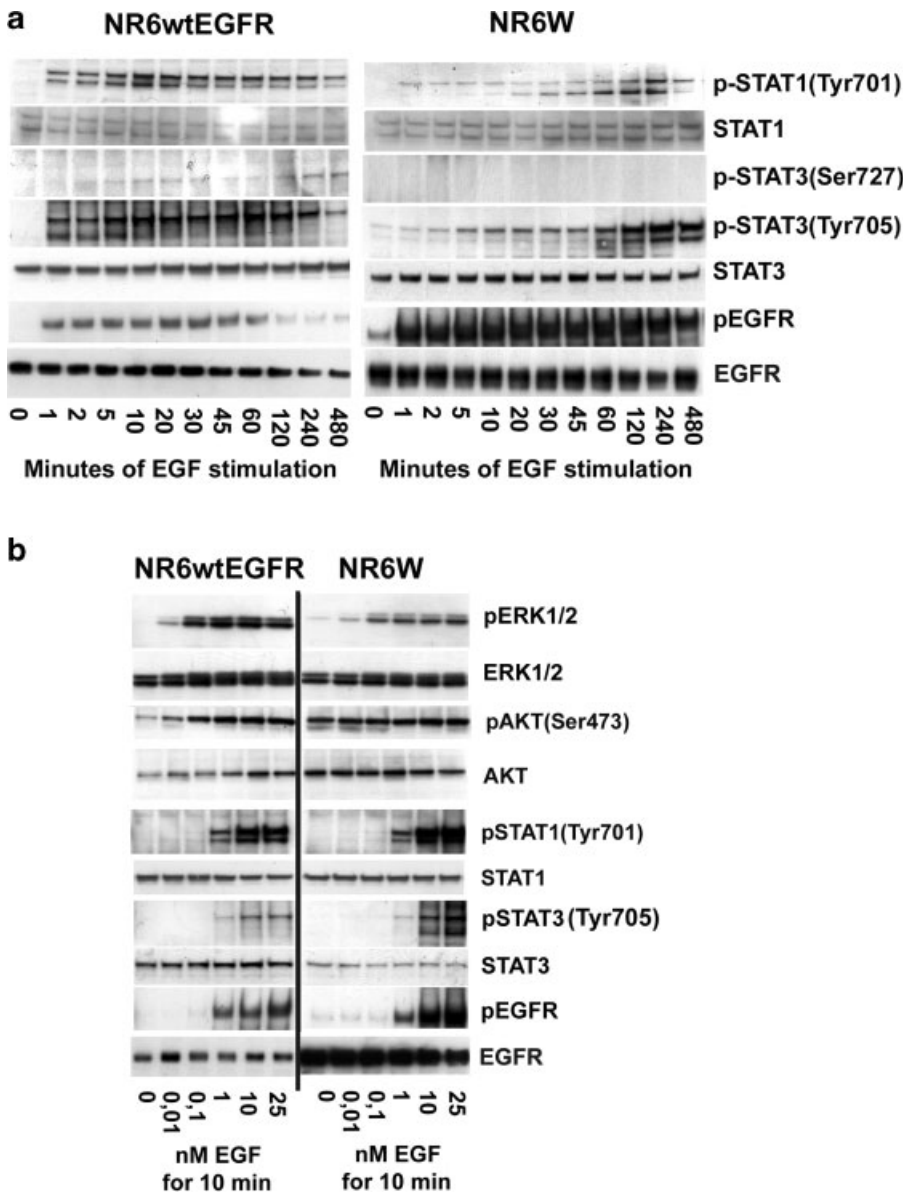


Fig. 7. **a**: Time course of STAT1 and STAT3 phosphorylations induced by 10 nM EGF in the NR6wtEGFR and NR6W cell lines. **b**: The effects of EGFR activation level on the activation of STAT1 (Tyr701), STAT3 (Tyr705), ERK1/2, and AKT (Ser473) were investigated in the NR6wtEGFR and NR6W cell lines upon stimulation with different concentrations of EGF. Experiments were repeated at least three times and representative immunoblots are shown.

This study also led to the identification of many novel EGF inducible genes. Some of these were induced by EGF in both the high and low EGFR expressing cell lines, while some were unique for either EGFR expression level. Many of these genes are immediate early genes encoding transcription factors and have previously been identified as serum and growth factor regulated genes [Deleu et al., 1999; Fambrough et al., 1999; Iyer et al., 1999]. The reason for the differential regulation by high and low levels of EGFR probably relate to different signaling kinetics, as the receptors in the low EGFR expressing cell line seem to induce signaling and gene expression faster compared to the EGFRs in the NR6W cell line (Figs. 5c and 7a). A feasible explanation is that the EGFRs in the NR6W cell line are desensitized to EGF, a common event in high EGFR expressing cells [Northwood and Davis, 1990].

To date, only limited information is available on the EGFR regulated transcriptome. However, two studies have investigated the effect of EGF stimulation of NIH-3T3 fibroblasts (6,500 genes) and MDA-MB-361 cells (6,000 genes), respectively, and many changes in gene expression are rediscovered in the present study [Fambrough et al., 1999; Sweeney et al., 2001].

In addition to our previous study [Pedersen et al., 2001b], one gene expression study has investigated the changes in gene expression induced by expression of EGFRvIII in two glioblastoma cell lines: D54-EGFRvIII and U87MG-ΔEGFR [Lal et al., 2002]. Using serial analysis of gene expression (SAGE), this study identified 70 transcripts induced by EGFRvIII expression. Similarly, our previous study identified 125 genes whose expression were changed more than a factor 2 by EGFRvIII expression in a small cell lung cancer cell line (GLC3) [Pedersen et al., 2001b]. Although apparently only few genes are found to overlap between the three studies, we do not interpret this as a general discrepancy as it is very difficult to compare SAGE and oligonucleotide array expression data. Furthermore, the three studies analyzed different numbers of genes and used different experimental conditions [Pedersen et al., 2001b; Lal et al., 2002]. The advantage of the NR6 model system is its "pureness" as NR6wtEGFR and NR6W only express wild-type EGFR and the fully tumorigenic NR6M only expresses the variant EGFRvIII, which is required for its neoplastic phenotype. Hence, the only differences between cell types should be the receptor and their expression levels and their corresponding influence on intracellular signaling and thus gene expression.

Role of the Interferon Responsive Genes

A significant finding of this study was that one third of the genes induced by ligand-activated EGFR at both expression levels, but not EGFRvIII, consisted of previously reported interferon responsive genes and in particular interferon-γ responsive genes. Induction of this module of genes has not previously been attributed to EGFR activation, but other studies have shown that a mutant version of the platelet derived growth factor receptor (PDGFR) and the ABL1 tyrosine kinase/breakpoint cluster region (BCR) fusion oncogene BCR/ABL1 are able to induce a similar gene expression profile [Fambrough et al., 1999; Hakansson et al., 2004]. The induction of four selected genes in this module (*Socs3*, *Irf1*, *IIGP*, and *Ifi47*) was more rapid and transient in the low EGFR expressing cell line, while no expression of these genes was observed in NR6M. Moreover, release from the inhibitor AG1478 did not result in induction of the genes in this cell line, ruling out the possibility of compensatory downregulation of the genes due to the constitutive nature of the receptor.

So what is the significance of this activation by EGFR and lack of it by EGFRvIII? Possible clues come from the functions of genes in the module and the normal function of interferons. Interferons are small cytokines with antiviral, antitumor, and immunomodulatory properties [Tannenbaum and Hamilton, 2000; Ikeda et al., 2002]. The chemokine (C-X-C motif) ligand 10 (IP-10) has been demonstrated to inhibit EGF induced cell motility, while the suppressor of cytokine signaling factors SOCS-1 and SOCS-3 has been found to negatively regulate EGFR activation possibly by inducing ubiquitination-dependent EGFR degradation upon ligand binding [Shiraha et al., 1999; Xia et al., 2002]. It is likely that the products of the genes in this module are involved in a negative feedback mechanism abrogating or limiting the tumor promoting effects of overactivated EGFR. Another possibility stems from the fact that many products of the genes in the interferon module including the 47-kDa GTPases IRG-47 (*Ifi47*), GTPI (*AI481100*), IIGP1 (*AW111922*), IGTP (*Igtp*), and TGTP (*Tgtp*) are known to play roles in host resistance to pathogens [Boehm et al., 1998; Collazo et al., 2001]. These genes may play a role in modulating the immune response, perhaps by alerting the immune system that a cell has unregulated growth factor receptor signaling. The lack of induction of this module by EGFRvIII suggests that this growth factor receptor/tumor inhibiting mechanism is absent or insufficient downstream of EGFRvIII.

STAT1 and STAT3 Signaling Correlates With Induction of the Interferon Responsive Genes

In this study, we also sought a possible explanation for the observed induction of interferon responsive genes by ligand-activated EGFR. Upon binding of interferons to their cognate receptors, they initiate a signaling cascade, involving the Janus kinase family and the STAT family of transcription factors,

leading to transcriptional induction of the interferon stimulated genes [Platanias and Fish, 1999]. EGFR has previously been found to induce phosphorylation of STAT1 and STAT3 wherefore EGFR mediated activation of STATs could be a feasible explanation of the observed induction of interferon responsive genes in these cell lines [Grandis et al., 1998, 2000; Kloth et al., 2002]. Indeed, it was found that ligand-activated EGFR in both the high and low EGFR expressing cell lines induced phosphorylation of STAT1 and STAT3, which were dependent on EGFR tyrosine kinase activity. While, EGFRvIII was unable to induce phosphorylation of these STATs under all conditions. Thus, the activation of STAT1 and STAT3 by EGFR correlates well with the induction of the interferon responsive genes. The generality of these results were confirmed in the high EGFR expressing human epidermoid carcinoma cell line A431 and in the EGFRvIII expressing glioblastoma cell line U87MG-ΔEGFR. The role of STAT1 and STAT3 downstream of EGFR is unsettled. However, generally activation of STAT1 is associated with growth arrest and apoptosis, while activated STAT3 appears to have the opposite effects [Grandis et al., 1998, 2000; Bromberg, 2001]. Here the phosphorylation level of STAT1 more closely follow the induction of the interferon responsive genes as compared to STAT3, suggesting that STAT1 may be the major activator of this module of genes.

It has also been reported that ligand concentration is important for the level of STAT1 phosphorylation, whereas STAT3, ERK, and AKT seem less dependent [Habib et al., 2003]. In this study, though, both STAT1 and STAT3 phosphorylations depend on relatively high ligand concentrations of EGF (1 nM) and thus on high receptor phosphorylation. In contrast to STAT1 and STAT3, both AKT and ERK phosphorylations are induced by low concentrations of EGF (0.1 nM) in the low EGFR expressing cell lines. Whereas the high EGFR expressing cell line has constitutively phosphorylated AKT and attenuated ERK phosphorylation, not changing significantly in response to EGF. This could explain why EGFRvIII does not induce STAT phosphorylations, as the low level of constitutive EGFRvIII phosphorylation observed in NR6M and U87MG-ΔEGFR may be insufficient for inducing STAT1 and STAT3 phosphorylations, although sufficient for activation of other signaling molecules such as ERK, PLC-γ, and AKT [Lorimer and Lavictoire, 2001; Narita et al., 2002; Pedersen et al., 2004].

An interesting general principle emerging from this study is that high expression of EGFR leads to spontaneous activation of EGFR, constitutive signaling, constitutive expression of tumor promoting genes, and hence transformation. Upon stimulation with EGF, STAT1 and STAT3 become activated, leading to sustained induction of the interferon responsive genes and growth arrest. EGFRvIII mimics overexpressed EGFR, although due to its lost ability to bind ligand, it is unable to reach a phosphorylation state sufficient for STAT activation. We can, however, not exclude that this lack of activation is independent of EGFRvIII expression level, although the lack of phosphorylations in both NR6M and the high EGFRvIII-expressing cell line U87MG-ΔEGFR indicate it.

CONCLUSIONS

Based on the interferon gamma module and the signaling to the STAT proteins, it seems likely that EGFRvIII only regulates a subset of signaling pathways, and thus expression of genes, of those regulated by ligand-activated EGFR. The lack of induction of this module by EGFRvIII suggests that this cancer-specific receptor may lack an important growth inhibitory response due to its low level of activation. Such a response may only be relevant in vivo, where interactions with other cells, in particular immune cells, are important.

ACKNOWLEDGMENTS

Supplementary information is available at www.radiationbiology.dk.

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