EGFR induces expression of IRF-1 via STAT1 and STAT3 activation leading to growth arrest of human cancer cells

Peter Andersen,[1] Mikkel W. Pedersen,[1] Anders Woetmann,[2] Marie-Thérése Stockhausen,[1] Niels Ødum[2] and Hans S. Poulsen[1]

[1]Department of Radiation Biology, Section 6321, Finsen Center, Copenhagen University Hospital, Copenhagen, Denmark

[2]Institute of Molecular Biology and Physiology, Department of Immunology, Panum, building 22.5, Blegdamsvej 3C, DK-2200 Copenhagen N, Denmark

e-mail: peter.andersen@rh.regionh.dk

www.radiationbiology.dk

Background

- Overexpression of EGFR and the constitutively active EGFR variant EGFRvIII is frequently associated with human cancer
- We have previously demonstrated that ligand-activated EGFR induces expression of genes normally inducible by interferons, including interferon regulatory factor-1 (IRF-1)[1]
- IRF-1 is an important effector arm of the immune system as well as a regulator of the cell cycle and apoptosis

Aim

To investigate the role of IRF-1 in EGFR overexpressing cancer cells

Materials and Methods

- **Cell lines:** The human head and neck carcinoma cell line HN5, the human skin carcinoma cell line A431 – both overexpressing EGFR, and the two murine fibroblast cell lines NR6M – expressing EGFRvIII, and NR6 – not expressing any of the receptors
- **Quantitative RT-PCR:** Reactions were carried out using Trizol extracted RNA and SuperScript III Platinum Two-Step qRT-PCR Kit with SYBR Green (Invitrogen)
- **Immunoblot:** Protein lysates were resolved by SDS-PAGE, electroblotted onto nitrocellulose membranes, incubated with antibodies and visualized by ECL
- **Precipitation:** Protein lysates were mixed with biotin-conjugated oligonucleotide-probes, then mixed with avidin-agarose beads, washed and subjected to immunoblotting and incubated with antibodies to STAT1 and STAT3
- **MTT assay:** Cells were plated in 96 wells, serum-starved for 24 hours and incubated 72 hours prior to MTT addition
- **Flow cytometry:** Cells were stained with CFSE, 7-AAD and Annexin V conjugated PE and analyzed by flow cytometry

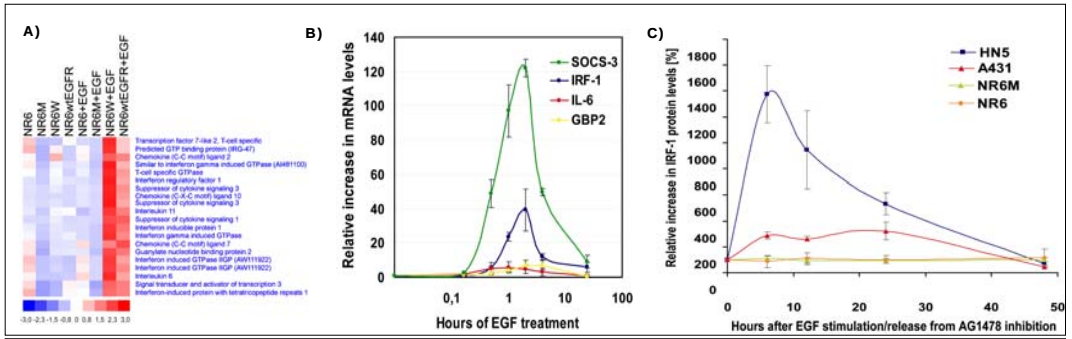


Fig. 1. The module of interferon-associated genes (modified from [1]). B) IRF-1, SOCS-3, IL-6, GBP-2 mRNA levels were detected in Trizol extracted RNA samples from EGF treated HN5 cells by quantitative real-time PCR analyses (similar expression pattern was observed in A431). C) Relative increase in IRF-1 protein levels. Bars indicate standard error of mean.

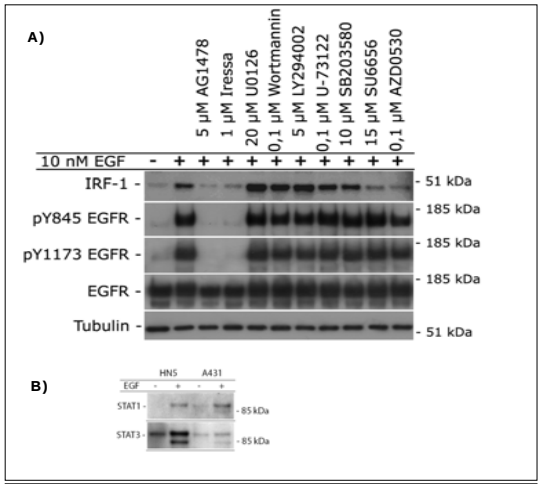


Fig. 2. A) Western blot analysis showing IRF-1 and EGFR levels as well as phosphorylation status of Y845- and Y1173EGFR in HN5 cells treated with the above mentioned inhibitors and/or with 10 nM EGF for one hour – SU6656 and AZD0530 are both potent Src kinase inhibitors (similar results were observed in A431) B) Western blot analysis showing STAT1 and -3 levels from biotin-conjugated GAS sequence precipitated cell lysates from HN5 and A431.

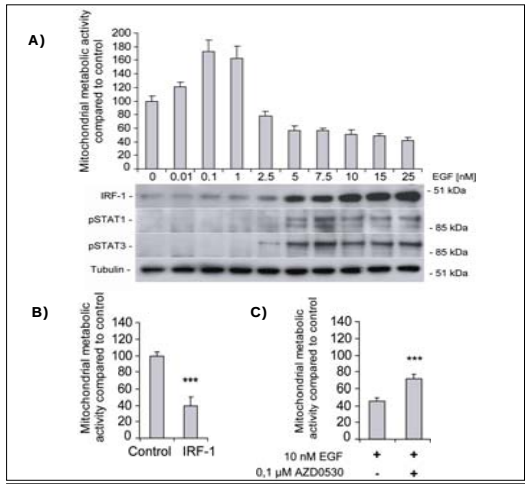
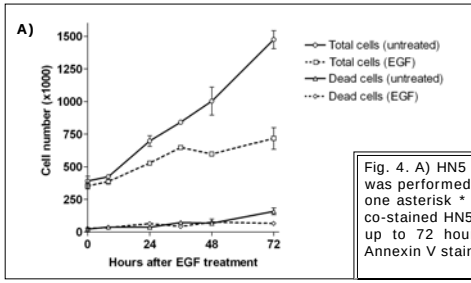


Fig. 3. A) Upper: HN5 cells treated with the indicated EGF concentrations for 72 hours. Lower: IRF-1 protein levels and phosphorylation status of STAT1 and -3 from HN5 cells treated with EGF for one hour. B) HN5 cells transfected with either pCMVBL/IRF-1 (IRF-) or empty vector (control) for 48 hours. C) HN5 cells treated with 10 nM EGF or with 10 nM EGF + 0,1 µM AZD0530. *** ($P<0.001$).



B)

	A431		HN5	
	Annexin V positive cells	Annexin V + 7-AAD positive cells	Annexin V positive cells	Annexin V + 7-AAD positive cells
Untreated	3,53 %	7,15 %	5,2 %	10,42 %
0,1 nM EGF	2,69 %	7,1 %	3,54 %	7,58 %
10 nM EGF	2,4 %	5,54 %	3,41 %	7,05 %

Fig. 4. A) HN5 cells treated with 10 nM EGF or left untreated for up to 72 hours. Student's *t*-test was performed on the data and statistical changes in cell number are indicated in the figures by one asterisk * ($P<0.02$) and two asterisks ** ($P<0.003$). B) Apoptosis in 7-AAD and annexin V co-stained HN5 and A431 cells treated with either 0.1 nM EGF, 10 nM EGF or cells left untreated up to 72 hours and measured by flowcytometry analysis. Early apoptosis is measured by Annexin V staining and late apoptosis by co-staining with 7-AAD and annexin V.

Results

- IRF-1 mRNA and protein levels were rapidly upregulated by activated EGFR, but not by EGFRvIII **(Fig. 1B and 1C)**
- The EGFR induced expression of IRF-1 was dependent on Src kinase activity **(Fig. 2A)**
- STAT1 and STAT3 binds to an activation sequence (GAS) from the IRF-1 promoter in response to EGF **(Fig. 2B)**
- EGFR-induced expression of IRF-1 correlated with phosphorylation of STAT1 and STAT3 **(Fig. 3A)**
- EGF increased expression of IRF-1 in a dose dependent manner that correlated with decreased cell viability **(Fig. 3A)**
- Overexpression of IRF-1 showed similar effects on cell viability **(Fig. 3B)**
- Inhibition of EGFR-induced IRF-1 expression with the potent Src inhibitor AZD0530 reduced the EGF-mediated growth inhibition **(Fig. 3C)**
- High concentrations of EGF significantly reduced cell proliferation, but did not induce apoptosis **(Fig. 4A and 4B)**

Conclusions

- The IFN-like module of genes is tightly regulated by EGFR in human cancer cells
- Activated EGFR, but not EGFRvIII induces expression of IRF-1
- The regulation is dependent on both EGFR tyrosine kinase activity and Src family kinase activity
- EGFR induces expression of IRF-1 via STAT1 and STAT3
- EGFR-induced IRF-1 is EGF-concentration dependent, and IRF-1 expression correlates with inhibition of cell proliferation

Reference:

[1] Pedersen MW. *et al. J Cell Biochem*. 2005;96(2):412–27