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Reduced inducibility of SOCS3 by interferon gamma associates with higher resistance of human breast cancer lines as compared to normal mammary epithelial cells

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The resistance to interferons (IFNs) limits their anticancer therapeutic efficacy. Here we studied the antiproliferative effect of interferon gamma in relation to SOCS3 expression in a panel of breast cancer cell lines and normal mammary epithelial cells. Compared to normal cells most breast cancer lines (7/8) were highly resistant to IFN-gamma. Using Northern blot and real time RT-PCR we investigated transcription of SOCS3 genes. All normal epithelial cells (4/4) showed SOCS3 induction (2-14 fold) while most breast cancer lines did not or weakly activated SOCS3 after the interferon gamma treatment. Among the cancer lines, the MDA-MB-468 cells showed increased sensitivity to IFN-gamma and relatively high level of SOCS3 induction (2-3 fold). Together, there was a good correlation ($P<0.001$) between SOCS3 inducibility and growth inhibition by IFN-gamma. The correlations between sensitivity and STAT1 phosphorylation levels (pY-701) were less pronounced since there were cases of STAT1-pY induction (2/12) without apparent growth inhibitory effect. The data indicate defective STAT1 phosphorylation and SOCS3 induction in breast cancer cells. These defects likely contribute to reduced sensitivity of malignant cells to antiproliferative effect of IFN-gamma.

Key words: suppressor of STAT signaling, STAT1, breast cancer, interferon, cell resistance

Interferons can inhibit growth and proliferation of different target cell populations and some of them are widely used in the therapy of various cancers [1, 2]. However, in solid tumors the effectiveness of the treatment is often unsatisfactory probably due to acquired and/or per se resistance to biological activity of IFNs. Several studies indicated importance of signal transducers and activators of transcription (STATs) in IFN-gamma signalization and resistance [3, 4, 5]. These proteins seem to be crucial components of JAK (Janus tyrosine kinase)/STAT signaling pathways that mediate specific external stimuli including interferons (IFNs) from cell surface to the nucleus. STATs comprise a multigene family (STAT1-6) of latent cytoplasmic factors with dual functions: transduction of the signal from cytoplasm to the nucleus and modulation of target gene transcription [for review see 6, 7]. Activation of STATs is dependent on conserved tyrosine phosphorylation, which induces dimerization (activation) of these proteins and their subsequent nuclear translocation. Overexpression of dominant negative STAT1 leads to the abrogation of antiproliferative effect of interferon gamma in melanoma cells [4] suggesting that activated STAT1 could act as a growth inhibitory molecule and tumor-suppressor protein [for review see 8, 9]. Several studies indicated defects of STAT1 activation and phosphorylation pathways in tumor cells [for review see 6, 10, 11]. In contrast, persistently activated STAT3 has been frequently detected in breast cancer derived cell lines [12, 13] and primary breast carcinomas [14, 15, 16] but not in normal mammary epithelial cells [13, 17] indicating its oncogenic behavior.

Attenuation or termination of cytokine signals is regulated at multiple steps by several groups of proteins [18, 19]. Out of them, endogenous suppressors of cytokine signaling (SOCSs) participate in the survival, differentiation, proliferation and apoptosis of nearly all cell types including mammary cells [for review see 20, 21, 22]. It has been suggested that SOCS1 and SOCS3 are critical inhibitors of IFN-gamma signaling

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[5, 23]. In support, the SOCS3 induction seems to be needed for antiproliferative effect of various cytokines including interferons in leukemia [24] and melanoma cells [25, 26]. On the other hand, these and other studies [27, 28] indicated that constitutive SOCS3 expression might confer growth advantage and resistance to various cytokine stimuli. Reports of aberrant DNA methylation of SOCS genes in a number of breast cancer cell lines and tumors [29, 30] have led to the suggestion that SOCS genes are silenced during oncogenic transformation of mammary tissue. In line with this supposition, decreased expression of SOCS3 has been positively correlated with increased metastatic potential in breast tumors [31]. On the other hand, constitutive expression of SOCS genes was reported in breast cancer lines [13] indicating absence of epigenetic transcriptional block. Of note, the SOCS3 expression was downregulated by exogenous cytokine stimuli in some cell lines. Thus, it seems that the role of SOCS proteins in IFN-mediated growth inhibition and/or breast cancer development/progression is complex and needs further investigation.

In this work, we have used a collection of 8 human breast cancer-derived cell lines and 5 primary cell cultures of normal mammary epithelium to investigate whether aberrant STAT/SOCS signaling might be involved in defective IFN-gamma response and antiproliferative effect of this cytokine in breast carcinoma. We asked the following questions: (i) Are there differences in the SOCS3 expression levels between breast cancer cells and normal mammary epithelium? (ii) How are the SOCS3 expression and STATs activation patterns influenced by IFN-gamma? (iii) What is the relationship between the antiproliferative effect of IFN-gamma, activation of STATs and expression of SOCS3? To address these questions, we examined the SOCS3 mRNA levels by quantitative real-time PCR and Northern blot hybridization; the protein posttranslational modifications were analyzed by Western blot. The molecular parameters were correlated with the antiproliferative effect of interferon gamma in a panel of normal and breast cancer cell lines. Evidence was obtained for correlation between SOCS3 inducibility, STAT1 phosphorylation and cytotoxic effect of IFN-gamma.

Materials and methods

Cell cultures. A collection of 8 human breast cancer-derived cell lines and 5 primary cell cultures of normal mammary epithelium (listed in Table 1) was used. Breast cancer cell lines were maintained in DMEM (GIBCO, USA) supplemented with 2 g/l of sodium bicarbonate, L-glutamine, insulin, antibiotics and 10% of fetal bovine serum. Cells were cultured in the incubator at 37°C in a humidified atmosphere with 5% CO_2.

Primary cell cultures of normal mammary epithelium were derived from reduction mammoplasty. The DMEM medium used for cultivation of normal cells was supplemented with all non-essential amino acids, sodium pyruvate, $NaHCO_3$, 10% bovine serum, 2% fetal bovine serum, hydrocortisone, insulin (Novo Nordisk), cholera toxin and EGF (Sigma, USA). The second to third passage of cells was used in experiments.

Growth inhibition assay. The antiproliferative effect of recombinant human IFN-gamma was assessed in all cell cultures. Cells were seeded into 96-well microplates at a density of 2000 cells per well. All analyses were performed in three independent experiments. The absorbance was measured in three parallel wells in each experiment. One day after seeding, the medium was replaced by medium containing IFN-gamma (Sigma, USA) at a concentration of 10 ng/ml and cultures were incubated at 37° C in a humidified CO_2 atmosphere. After 24, 48 and 72 h intervals of treatment, the WST-1 colorimetric assay (Roche, Mannheim, Germany) was performed according to the manufacturer's protocol. The growth inhibition (cytotoxicity) was expressed as a percentage of the growth of control untreated cells (Table 1).

Table 1 Characteristics of human mammary epithelial cells. Resistance to interferon gamma

Culture		Origin	IFN gamma resistance [a]
Normal cells	AN	NME	40-60
	RT	NME	30-50
	NME19+20	NME	50-70
	NME-26	NME	30-50
	NME35/2	NME	30-40
Cancer cell lines	T-47D	DC; M	80-100
	MCF-7	A; M	80-100
	BT474	DC	80-100
	ZR-75-1	DC; M	80-100
	CAMA-1	A; M	80-100
	MDA-MB-231	A; M	90-100
	MDA-MB-468	A; M	50-60
	SK-BR-3	A; M	80-100

Abbreviations: IFN = interferon; M = metastatic cells; A = adenocarcinoma; DC = ductal carcinoma; NME = primary cell culture of normal mammary epithelium from reduction mammoplasty (mixture of lobular and ductal cells)
[a] Expressed as percentage of viable cells after 72 h time interval of IFN gamma treatment as related to untreated controls. Range of cytotoxicity values obtained from at least three independent experiments.

IFN-gamma treatments. Activation dosage of IFN-gamma (10 ng/ml) was selected from the dose-response curve. Subconfluent cells were incubated with IFN-gamma for 30 min (in some cases, for 2, 8, 24, 48 and 72 hours) at 37°C in a humidified atmosphere with 5% CO_2. Control samples were incubated in parallel without IFN treatment.

Western blot analyses. The protein content in whole-cell extracts was determined by Bradford assay (Bio-Rad, Germany). Approximately 20 μg of total proteins were separated by SDS-PAGE (10% gels) and the proteins were transferred to

a nitrocellulose membrane (Bio-Rad, USA) Phosphorylated STAT1 protein was concentrated by immunoprecipitation prior to electrophoresis. After the primary antibody binding reaction, the blots were incubated with either anti-mouse or anti-rabbit horseradish peroxidase-conjugated secondary antibody (Amersham Biosciences, UK) and developed using the enhanced chemiluminiscence (ECL) detection system (Amersham Biosciences, UK) according to manufacturer's instructions.

The following primary antibodies were used: STAT1 anti-pY701 (Cell Signaling Technology, USA) and a polyclonal antiserum against C-terminal domain of STAT1 (S1C) raised in author's lab [32].

RNA analyses. Total RNA was isolated from frozen cell pellets using RNeasy kit (Qiagen, Hilden, Germany). For quantitative real-time PCR, each sample was extensively pre-treated with DNase I and then reverse transcribed using random nonamer primers (Sigma, USA) and Superscript II polymerase (Invitrogen, Carlsbad, CA, USA). The real-time PCR reaction was done on Rotor-Gene™6000 series real-time analyzer (Corbett Life Science, Sydney, Australia). The cDNAs were amplified using specific primer sets for SOCS3 and GAPDH (Superarray, USA), respectively. We used amplification conditions recommended by manufacturer of primers and RT² qPCR Master Mix (Superarray, USA).

In some cases, RT-PCR results were verified by Northern blot using a SOCS3 probe [33] as described in our previous study [34]. The blots were rehybridized with human 18S rDNA probe obtained by PCR amplification of a 300 bp subregion.

Statistical data analysis . Standard rank summary statistics were used to describe obtained data – median, min/max values. Non-parametric Mann-Whitney U test was applied to test differences between normal and malignant cell lines, based on comparison of median values. Correlation between SOCS3 induction and cell resistance was assessed both by parametric Pearson's correlation and by Spearman's rank correlation coefficient. A value p = 0.05 was taken as cut-off for statistical significance in all applied tests.

Table 2 Stimulation of STAT1 phosphorylation and SOCS3 expression after interferon gamma

Culture	SOCS3 induction (fold increase)[a]	STAT1 phosphorylation at Y-701[b]
AN	13.1	+++
RT	4.1	+++
NME19+20	1.9	++
NME-26	4.4	+++
NME35/2	n.d.	+++
T-47D	0.8	+
MCF-7	1.3	+++
BT474	1.0	+
ZR-75-1	1.1	+
CAMA-1	0.9	+
MDA-MB-231	2.4	++
MDA-MB-468	3.1	++
SK-BR-3	0.5	+/-

[a] The data are taken from the real-time PCR experiment (Figure 2B)
[b] The data are taken from the Western blot analysis (Figure 5); signal intensities were estimated by visual inspection of blots. n.d. – not determined.

Results

Differential sensitivity of cell cultures to IFN-gamma. Antiproliferative effect of IFN-gamma on the cell growth was studied in breast cancer-derived cell lines and primary cell cultures of normal mammary epithelium. The viable cells counts were assessed at the 0, 24, 48 and 72 hours time point intervals by a WST-1 test. The cell count data are summarized in Table 1. It is clear that IFN-gamma significantly retarded the growth of almost all cultures of normal epithelial cells (by 30-70%) while no growth inhibition was observed in breast cancer lines. Out of the cancer lines, the MDA-MB-468 cells were exception in being partially sensitive to IFN-gamma.

Stimulation of SOCS3 expression by IFN-gamma. We first studied the time dependence of SOCS3 mRNA induction by IFN-gamma. The MDA-MB- 468 and T47D breast cancer cells

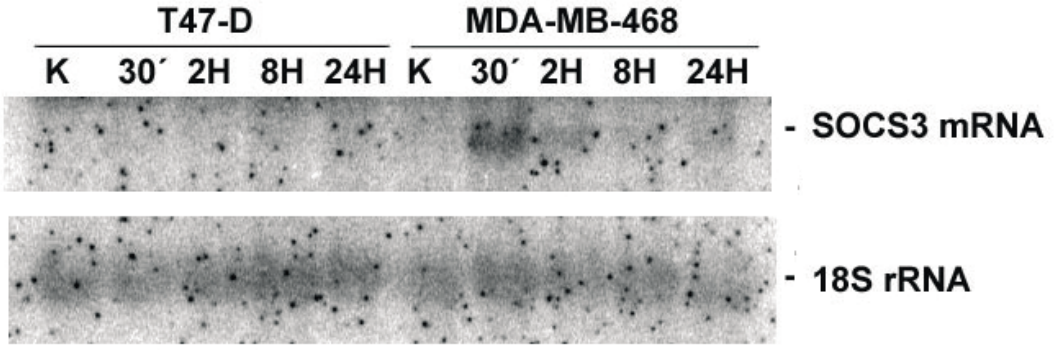

Figure 1 Northern blot analysis of time-dependence of SOCS3 induction. The cells were exposed to IFN-gamma (10ng/ml) for time point intervals indicated.

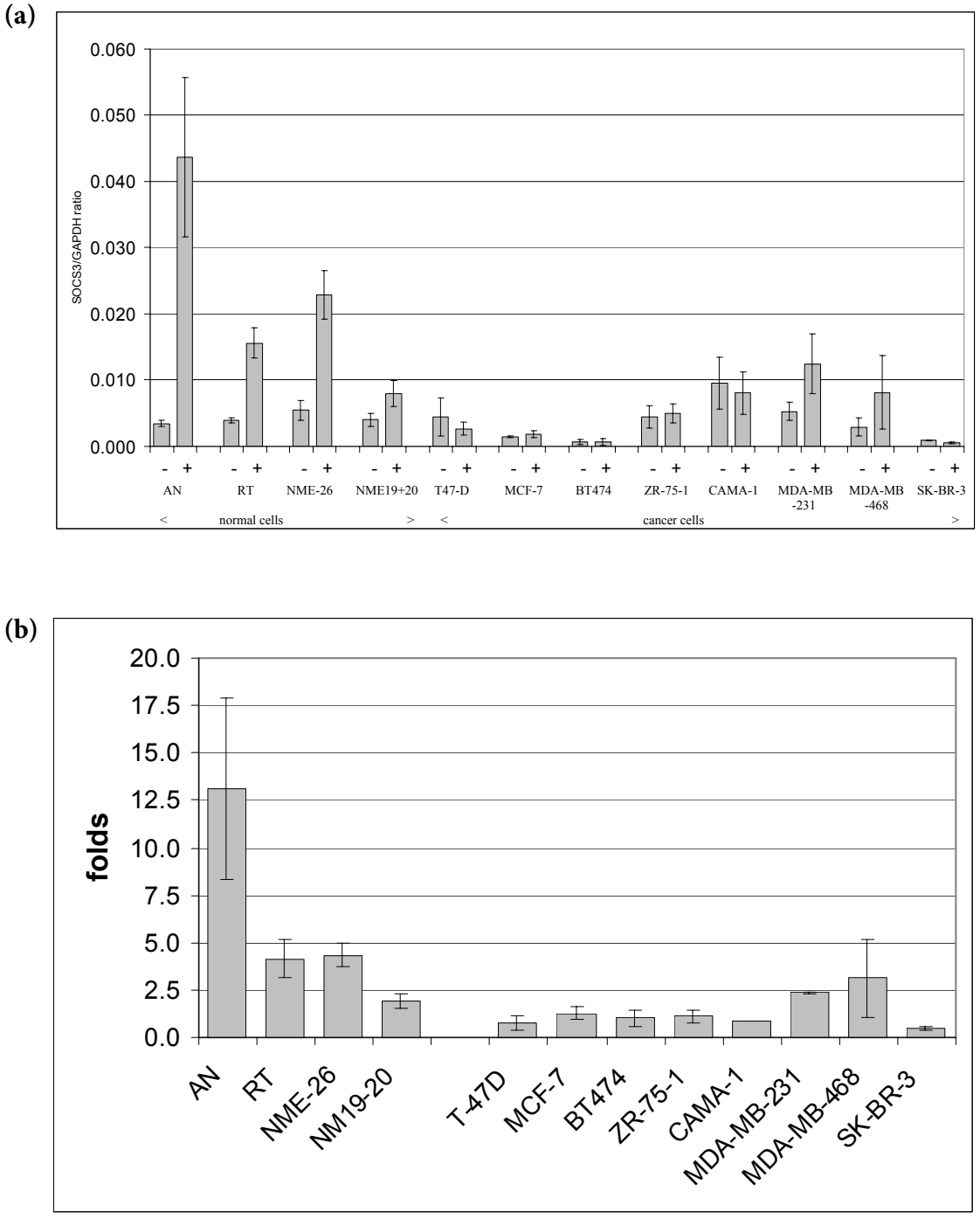

Figure 2 Real-time PCR analysis of SOCS3 transcripts in normal epithelium and breast cancer lines.

(a) SOCS3 transcripts normalized to GAPDH transcripts. The data were collected from 3-4 independent experiments and expressed as a mean ± s.d. (b) SOCS mRNA levels expressed as fold increases of IFN-treated over non-treated samples. (-) non-treated control; (+) IFN gamma treated.

were treated for 0.5, 2, 8, and 24 h with interferon-gamma and RNA was extracted from each interval. The Northern blot analyses revealed absence of the SOCS3-hybridization signal in both treated and non-treated T47D cells (Figure 1). In contrast, the 1.3-kb SOCS3 transcript was readily detected in MDA-MB-468 cells following the IFN-gamma stimulus.

Table 3. Summary statistics of examined parameters in normal and malignant cell lines

Cell lines	Cell resistance 1	Induction of SOCS3
	Median (minimum - maximum)	
Normal (n = 5)	40 (35 - 60)	4.3 (1.9 - 13.1)
Malignant (n = 8)	90 (55 - 95)	1.1 (0.5 - 3.1)
Total	90 (35 - 95)	2.4 (0.5 - 13.1)

[1] Entered data: mean of range obtained for each examined cell line

The duration of signal resembled the normal response, [35] reaching maximum within 30 min and slowly diminishing hereafter. These experiments indicated differences in the SOCS3 activation capacities between the lines and guided us to study the SOCS3 response at the quantitative level.

Using quantitative RT-PCR we determined the SOCS3 RNA levels in RNA samples from cells treated for 30 min with IFN-gamma. The data were expressed as normalized SOCS3 mRNA levels (Figure 2A) and folds of increase (Figure 2B). The basal levels of SOCS3 were low with the exception of the CAMA-1 cells that had relatively high SOCS3 levels. After the IFN treatments all normal lines showed elevated SOCS3 signals while there were no or marginal increase in most breast cancer cells (with the exception of MDA-MB-468). In some cases (e.g. T47D), even slight decreases were observed. The correlations between SOCS3 inducibility and antiproliferative effect of IFN-gamma shown in Table 3 have been statistically analysed. Data displayed in Figures 3 and 4 show that there is nearly clear dichotomy between normal and malignant cells in both parameters, i.e. the cell resistance and SOCS3 induction.

STATs protein phosphorylation status. The STAT1 phosphorylation levels were investigated in non-treated and IFN-treated cells using Western blot analyses. The proteins were immunoblotted with antibodies against the phosphotyrosine and phosphoserine residues. Examples of protein blots are shown in Figure 5 and the results summarized in Table 2. The immunoreactions with antibodies to non-phosphorylated determinants revealed constitutive expression of STAT1 protein in all cell lines. However, the signals derived from phosphorylated STAT1-Y701 (STAT1-pY, further on) were low or negligible in non-treated samples. IFN-gamma strongly increased STAT1 phosphorylation signals in normal cells while the increases were less pronounced or even absent in malignant cells.

Discussion

Induction of SOCS3 in cancer and normal cells lines. IFN-gamma is one of the best-known inducer of the SOCS3 gene transcription. Yet, in many pathological conditions, the induc-

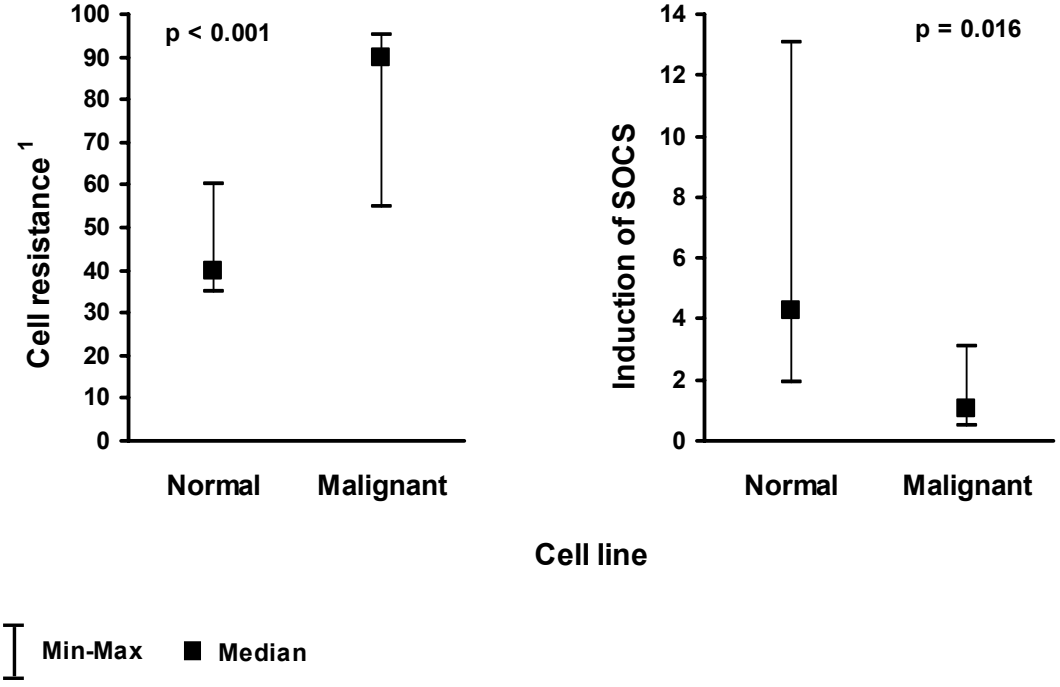

Figure 3 Cell resistance and induction of SOCS3 in normal and malignant cell lines.

p value: statistical significance evaluated by Mann-Whitney U test.

[1] Entered data: mean of range obtained for each examined cell line.

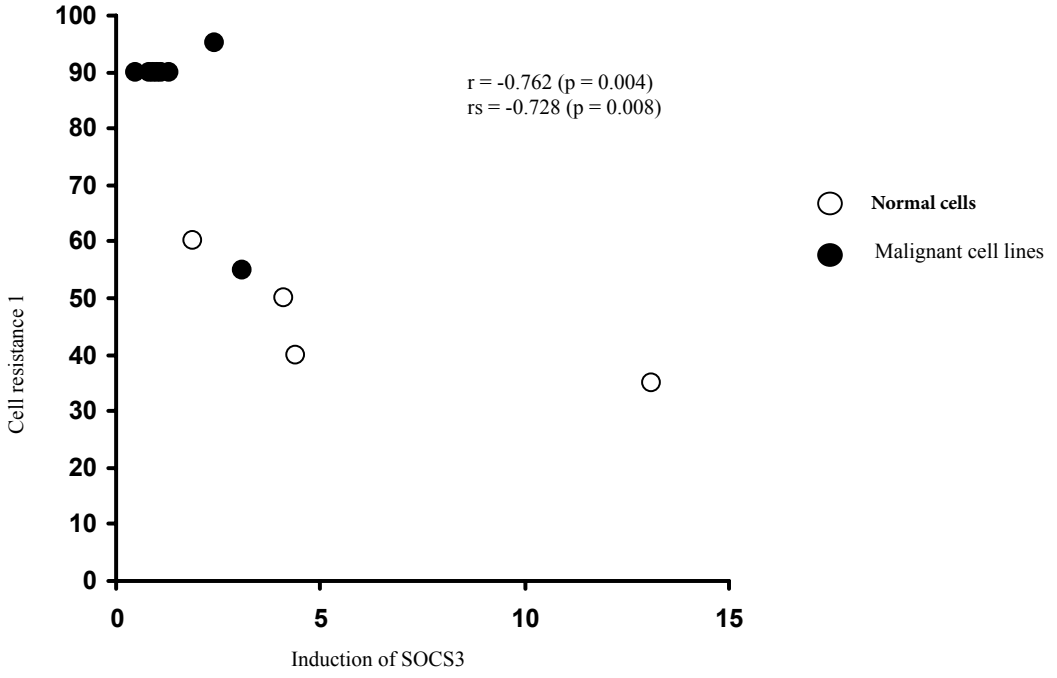

Figure 4 Correlation diagram between cell resistance and induction of SOCS3

[1] Entered data: mean of range obtained for each examined cell line, r_s = Spearman's rank correlation coefficient

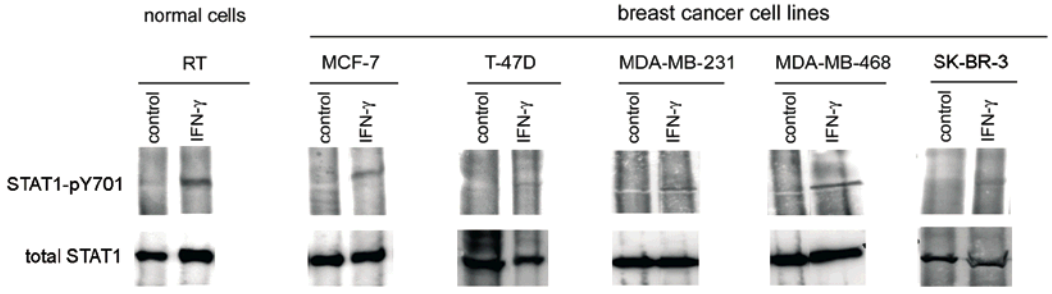

Figure 5 Activation of STAT1 signaling in IFN-gamma treated normal and breast cancer cells. Examples of Western blot analysis of STAT1 phosphorylation status. The antibodies against the Y701-phosphoform and total STAT1 recognized a ~90 kDa band.

tion fails from reasons that are not fully understood. In our experiments, SOCS3 was not significantly induced by IFN-gamma in about 63% of breast cancer lines and the remaining 37% lines showed significantly attenuated response. In contrast, non-malignant epithelial cells exhibited a robust induction of SOCS3 after the IFN-gamma treatment. These data pointed to specific defects in the JAK/STAT signal transduction pathways in some breast tumors, at least. We therefore investigated phosphorylation status of STAT1 protein discovering some correlations between activation and induction of SOCS3. For example, T47D cells weakly expressing SOCS3 had low STAT1-pY induction after IFN-gamma. Conversely, the MDA-MB-468 cells strongly induced both STAT1-pY and SOCS3 after the interferon stimulation. These correlations were, however, not strict. For example, IFN-gamma did activate STAT1-pY in MCF-7 cells without concomitant induction of SOCS3. Thus it appears that failure of SOCS3 activation could originate from various genetic and epigenetic lesions:

(i) There might be weak STAT1 signaling resulting in an insufficient activation of SOCS3 transcription. In this context,

there were two STAT1 binding sites identified in the SOCS3 promoter [36]. This is a likely reason for weak or negligible SOCS3 induction in most "non-responsive" breast cancer lines (Table 2). However, the duration of SOCS3 activation in MDA-MB-468 was similar to that of normal cells [35] but significantly shorter than in melanoma [25, 26]. Perhaps, the SOCS3 half-life could differ among different cell types.

(ii) The SOCS3 gene could be inactivated epigenetically. In these cases, SOCS3 activation would not occur even in the presence of STAT1 transactivation signals. The MCF-7 cells probably fall into this category since the SOCS3 promoter was shown to be inactivated by DNA methylation in this line [29, 30].

(iii) There might be a constitutive activation of SOCS3 masking its inducibility. In melanoma cells high basal levels of SOCS3 were negatively correlated with the induction by IFN-gamma [25] and interleukin 10 [26]. Relatively high (3-fold over the average) basal SOCS3 levels could be a possible reason for SOCS3 induction failure in the CAMA-1 line. Of note, the inverse correlation between high basal SOCS3 level and its inducibility is also apparent among normal cells: out of the four lines the AN cells had the lowest basal levels and highest induction magnitude.

Relationship between SOCS3 induction and interferon gamma cytotoxicity. IFN-gamma exhibits antiproliferative and pro-apoptotic properties in a number of cell types. Yet, large differences exist in the sensitivity among different cell lineages. Here, we studied expression of SOCS3 in relation to IFN-gamma mediated growth inhibition in several normal cells and breast cancer lines. We found that while normal epithelial cells were relatively sensitive (30-70% resistance) most breast cancer cells were highly resistant (90-100%) to IFN-gamma. The strong SOCS3 induction was observed in normal cells. The MDA-MB-468 cells were exceptional among cancer cells in having relatively "normal" SOCS3 activation and reduced sensitivity to IFN-gamma. Degree of cell resistance significantly and negatively correlated with SOCS3 induction and the correlation was confirmed by parametric Pearson's coefficient as well as by rank correlation (both with $p < 0.001$). The profile of points in correlation diagram (Figure 4) suggests that there cannot be necessarily only simple line relationship, some type of hyperbolic or polynomial function can be hypothesized. Confirmation of this feature however requires further research with more cell lines.

The data showed apparent similarities with other studies of molecular basis of cytokine sensitivity [24, 25, 26] allowing some generalization of our findings. First, cells that do not activate SOCS3 are usually resistant to IFN-gamma. A lack of SOCS3 inducibility might therefore be a marker of cell resistance. Second, constitutively activated SOCS3 does not confer sensitivity of cells to IFN-gamma. Third, STAT1-pY701 activation seems to be needed for SOCS3 activation and growth inhibition. Taken together, active STAT1 signaling and induction of SOCS3 seem to be required for manifestation of cytotoxic properties of interferon gamma in breast cancer cells.

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