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Network Analysis of *icb-1* Gene Function in Human Breast Cancer Cells

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ABSTRACT

Icb-1 is a human gene previously described by our group to exert important functions in cancer cells of different origin. We now performed microarray-based gene expression profiling with subsequent network modeling to further elucidate the role of *icb-1* in breast cancer cells. Analyzing the effect of *icb-1* knockdown on the transcriptome of MCF-7 cells, we found 151 differentially expressed genes exhibiting more than twofold changes, 97 of which were up- and 54 downregulated. Most of the upregulated genes were cancer-related genes associated with poor prognosis, invasion and metastasis, building an oncogenic network of TNF target genes. On the other hand, network analysis identified the downregulated genes to be primarily involved in interferon signaling and cellular apoptosis. Confirming these network data, we observed that cells with reduced levels of icb-1 exhibited an impaired response to the apoptosis inducers tamoxifen, staurosporine, actinomycin, and camptothecin. The data of this study suggest that icb-1 might exert a tumor-suppressor function in breast cancer and that its loss might confer relative resistance of breast cancer cells to apoptotic drugs. J. Cell. Biochem. 113: 2979–2988, 2012. © 2012 Wiley Periodicals, Inc.

KEY WORDS: DNA MICROARRAY ANALYSIS; ICB-1 GENE; BREAST CANCER CELL; APOPTOSIS; GENE EXPRESSION; GENE NETWORK

Human gene *icb-1* (C1orf38, *chromosome 1 open reading frame 38*) has been originally cloned and described by our group to be involved in differentiation processes of gynecological cancer cells [Treeck et al., 1998]. Recently, it has been identified as member of a new metazoan gene family called THEMIS coding for cytosolic proteins binding to the Grb2 adaptor protein involved in receptor tyrosine kinase signaling [Peirce et al., 2010]. Whereas leukocytes exhibit the highest expression levels of icb-1, this gene is also expressed in a variety of human tissues and cancer cell lines including breast cancer cells. We found icb-1 expression to be elevated after exposure of cancer cells to stimuli of cellular differentiation, like endometrial cancer cells to basement membrane or leukemia cells to retinoic acid [Treeck et al., 1998, 2002]. Data of a recent study suggested icb-1 to be a component of signaling pathways mediating differentiating effects of vitamin D3 and ATRA on breast cancer cells [Haselberger et al., 2011]. These reports clearly suggested that *icb-1* might be involved in such processes of cancer cells. Other studies demonstrating that *icb-1* is an interferon-gamma responsive gene which in turn inhibits the effects of this cytokine on tumor cells showed that the view on *icb-1* function had to be broadened [Treeck et al., 2005]. This was confirmed by identification of an estrogen response element (ERE) in the promoter region of this gene regulating it in an ERα-dependent manner [Bollmann et al., 2008]. Recently we reported icb-1 gene to affect estrogen responsiveness of ovarian and breast cancer cells [Konwisorz et al., 2010]. The proposed role of icb-1 in cancer cells was further corroborated by results suggesting that single nucleotide polymorphisms in this gene affect breast cancer susceptibility [Springwald et al., 2009].

To further approach *icb-1* function in breast cancer cells, in this study we performed DNA microarray analyses of MCF-7 breast cancer cells stably expressing icb-1 shRNA to identify gene networks affected by this gene.

Conflicts of interest: None.

Additional supporting information may be found in the online version of this article.

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MATERIALS AND METHODS

MATERIALS

Phenol red-free DMEM culture medium was obtained from Invitrogen (Karlsruhe, Germany), FCS was purchased from PAA (Pasching, Austria). MCF-7 breast cancer cells were obtained from American Type Culture Collection (Manassas). Camptothecin, staurosporine, actinomycin, TNFα, and LPS were from Sigma (Sigma, Deisenhofen, Germany). M-MLV-P reverse transcriptase and Caspase-Glo 3/7 kit were purchased from Promega (Mannheim, Germany). RNeasy Mini Kit, RNase Free DNase Set, and Quantitect SYBR Green PCR Kit were obtained from Qiagen (Hilden, Germany). PCR primers were synthesized at Metabion (Planegg-Martinsried, Germany). Transfectin reagent was obtained from BioRad (Hercules). Platinum Pfx Polymerase and OptiMEM medium were purchased at Invitrogen (Karlsruhe, Germany). SureSilencing shRNA plasmid for human *icb-1* (C1orf38) gene was purchased from SABiosciences, Frederick.

MCF-7 CLONES, CELL VIABILITY, AND APOPTOSIS ASSAYS

MCF-7 breast cancer cells were maintained in DMEM/F12 medium supplemented with 10% FCS, 1 mM sodiumpyruvate, and 10 ng/ml insulin. Cells were cultured with 5% CO_2 at 37°C in a humidified incubator. Generation of stable MCF-7 (icb-1 knockdown, icb-1 KD) and MCF-7 (control) cell lines by transfection with shRNA plasmids was described previously [Konwisorz et al., 2010]. Both MCF-7 lines were seeded in 96-well plates in triplicates (1,000 cells/well), and were treated with different concentrations of camptothecin, staurosporine, or actinomycin. After 24, 48, and 72 hours, relative numbers of viable cells were measured in comparison to the untreated control and the solvent control using the fluorimetrical, resazurin-based Cell Titer Blue assay (Promega) according to the manufacturer's instructions at 560Ex/590Em nm in a Victor3 multilabel counter (PerkinElmer, Germany). Cell viability was expressed as percentage of the vehicle control.

For determination of cellular apoptosis, MCF-7 cells were seeded in 96-well plates (5×10^3 cells/well) and treated with different concentrations of camptothecin, staurosporine, or actinomycin. After 4 h of treatment, cellular apoptosis was determined by measurement of caspase 7 activity by means of the luminometric Caspase-Glo 3/7 assay (Promega) according to the manufacturer's protocol using a Victor3 multilabel counter (PerkinElmer, Germany). Cellular apoptosis was expressed as percentage of the vehicle control or as percentage of the control-transfected cells. Statistical analysis of the data was performed by one-way ANOVA using Prism 2.0 Software (Graph pad, San Diego), with statistical significance accepted at $P < 0.05$.

REVERSE TRANSCRIPTION AND QPCR

Total RNA from human breast cancer cell lines was isolated by means of the SV Total RNA Isolation System (Promega) according to the manufacturer's instructions. From 0.3 μg total RNA, cDNA was synthesized using 100 U M-MLV-P reverse transcriptase (Promega), 2.5 mM dNTP mixture and 50 pM random primers (Invitrogen). For *real time* PCR detection of gene expression in an intron-spanning manner (primer sequences in Suppl.1), 2 μl cDNA were amplified using Light Cycler® FastStart DNA master mix SYBR Green I and the LightCyler 2.0 PCR device (Roche Diagnostics, Mannheim, Germany). The PCR program was 95°C for 15 min, followed by 45 PCR cycles (95°C for 10 s, 56°C for 30 s, 72°C for 30 s) and a final extension for 5 min at 72°C, followed by a standard melting curve analysis. In all RT-PCR experiments, a 190 bp β-actin fragment was amplified as reference gene using intron-spanning primers actin-2573 and actin-2876. Data were analyzed using the comparative $\Delta\Delta C_T$ method [Livak & Schmittgen, 2001] calculating the difference between the threshold cycle (C_T) values of the target and reference gene of each sample and then comparing the resulting ΔC_T values between different samples. In these experiments, mRNA not subjected to reverse transcription was used as a negative control to distinguish cDNA and vector or genomic DNA amplification.

GENECHIP™ MICROARRAY ASSAY

Processing of four RNA samples (two biological replicates from control-shRNA or icb-1-shRNA transfected MCF-7 cells) was performed at the local Affymetrix Service Provider and Genomics Core Facility, "KFB—Center of Excellence for Fluorescent Bioanalytics" (Regensburg, Germany; www.kfb-regensburg.de). Sample preparation for microarray hybridization was carried out as described in the Affymetrix GeneChip® Whole Transcript (WT) Sense Target Labeling Assay manual. Three hundred nanograms of total RNA were used to generate double-stranded cDNA, omitting the initial ribosomal RNA (rRNA) reduction procedure. Subsequently synthesized cRNA (WT cDNA Synthesis and Amplification Kit, Affymetrix) was purified and reverse transcribed into single-stranded (ss) DNA. After purification, the ssDNA was fragmented using a combination of uracil DNA glycosylase (UDG) and apurinic/apyrimidinic endonuclease 1 (APE 1). Fragmented DNA was labeled with biotin (WT Terminal Labeling Kit, Affymetrix), and 2.3 μg DNA were hybridized to the GeneChip Human Gene 1.0 ST Array (Affymetrix) for 16 h at 45°C in a rotating chamber. Hybridized arrays were washed and stained in an Affymetrix Washing Station FS450 using preformulated solutions (Hyb, Wash & Stain Kit, Affymetrix), and the fluorescent signals were measured with an Affymetrix GeneChip® Scanner 3000-7G.

MICROARRAY DATA ANALYSIS

Summarized probe signals were created by using the RMA algorithm in the Affymetrix GeneChip Expression Console Software and exported into Microsoft Excel. Data was then analyzed using Ingenuity IPA Software (Ingenuity Systems, Stanford) and the GeneMANIA prediction server [Warde-Farley et al., 2010]. Genes with more than twofold changed mRNA levels after icb-1 knockdown in both biological replicates were considered to be differentially expressed and were included in the analyses.

RESULTS

TRANSCRIPTOME ANALYSIS

Analyzing the effect of *icb-1* knockdown on the transcriptome of MCF-7 cells by means of GeneChip Human Gene 1.0 ST DNA microarrays (Affymetrix), we found 151 differentially expressed genes exhibiting more than twofold changes, 97 of which were

TABLE I. Gene Expression After Knockdown of *icb-1* Expression in MCF-7 Breast Cancer Cells as Assessed on the mRNA Level by Means of DNA Microarray Analysis (Affymetrix Chip)

Gene symbol	Gene name	Regulation (-fold)
Upregulated genes		
MUCL1	mucin-like 1	9.68
KRT81	keratin 81	6.66
CEACAM6	carcinoembryonic antigen-related cell adhesion molecule 6	5.50
S100A8	S100 calcium binding protein A8	5.10
CEACAM5	carcinoembryonic antigen-related cell adhesion molecule 5	4.54
KRT23	keratin 23 (histone deacetylase inducible)	4.21
MID1	midline 1	4.21
CAMK2D	calcium/calmodulin-dependent protein kinase II delta	4.07
GPX2	glutathione peroxidase 2 (gastrointestinal)	3.74
GALNT5	UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 5 (GalNAc-T5)	3.73
IGFBP5	insulin-like growth factor binding protein 5	3.68
UGT2B7	UDP glucuronosyltransferase 2 family, polypeptide B7	3.62
KRT6A	keratin 6A	3.56
MUC1	mucin 1, cell surface associated	3.36
KRT10	keratin 10	3.35
Downregulated genes		
DDX58	DEAD (Asp-Glu-Ala-Asp) box polypeptide 58	−2.85
IFI27	interferon, alpha-inducible protein 27	−2.91
SP110	SP110 nuclear body protein	−2.91
PARP9	poly (ADP-ribose) polymerase family, member 9	−2.92
SAMD9	sterile alpha motif domain containing 9	−2.96
NPY1R	neuropeptide Y receptor Y1	−2.98
OAS1	2′,5′-oligoadenylate synthetase 1, 40/46kDa	−3.09
OAS2	2′-5′-oligoadenylate synthetase 2, 69/71kDa	−3.19
TFPI	tissue factor pathway inhibitor	−3.55
MX2	myxovirus (influenza virus) resistance 2 (mouse)	−3.76
DDX60L	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60-like	−4.05
IFI44L	interferon-induced protein 44-like	−4.34
IFI44	interferon-induced protein 44	−4.71
DDX60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	−4.84
IFIT1	interferon-induced protein with tetratricopeptide repeats 1	−5.48
XAF1	XIAP associated factor 1	−7.06

Shown are the top 15 up- and downregulated genes.

up- and 54 downregulated. The three genes exhibiting the strongest decrease of mRNA levels were XAF-1 (XIAP-associated factor 1), IFIT1 (interferon-induced protein with tetratricopeptide repeats 1) and DDX60 (DEAD (Asp-Glu-Ala-Asp) box polypeptide 60). The three top upregulated genes were MUCL1 (mucin-like 1), KRT81 (keratin 81), and CEACAM 6 (carcinoembryonic antigen-related cell adhesion molecule 6). The top fifteen up- and downregulated genes are shown in Table I.

To verify the DNA microarray results, we performed qPCR analysis of 10 selected genes. PCR data confirmed the observed increase or decrease of mRNA levels after knockdown of icb-1 gene in MCF-7 breast cancer cells (Fig. 1). Transcript levels of MUCL1 were even stronger, about 19-fold elevated in qPCR analyses, the same was true for CEACAM5 and 6, which were increased 8- or 10.5-fold, respectively. Levels of CAMK2D mRNA, similarly to the microarray data, were about fivefold elevated, whereas increase of KRT81 expression after icb-1 knockdown was observed to be smaller, but still 2.5-fold. With regard to the downregulated genes, the status of XAF1 as gene with the strongest expression decrease was confirmed by qPCR. We also verified the observed downregulation of IFI44, DDX60, OAS1, and TFPI.

NETWORK ANALYIS

Using Ingenuity Pathway Analysis Software (Ingenuity Systems, Stanford), we analyzed the obtained microarray data. IPA software allowed prediction of biological networks and pathways associated with *icb-1* function as well as associated biological functions with regard to diseases and disorders, molecular and cellular functions and with regard to development and function of physiological systems (Table II). Furthermore, these analyses allowed identification of two gene networks affected by knockdown of icb-1 gene, a network of downregulated genes and a second network of induced genes. The downregulation network mainly consisted of interferon responsive genes known to be regulated by interferon regulatory factor 7 (IRF7) (Fig. 2). The induced network of cancer-related genes was predicted to be regulated by tumor necrosis factor (TNF) and transforming growth factor β1 (TGFB1) (Fig. 3).

INTERACTION OF ICB-1 AND TNF EXPRESSION

Given that pathway analysis identified TNF as a central trigger for induction of the cancer gene network and icb-1 previously has been reported to mediate LPS-triggered activation of TNF in macrophages [Peirce et al., 2010], we additionally examined expression of this cytokine by means of qPCR analysis. Whereas TNF expression in MCF-7 (icb-1 KD) cells was not significantly changed in the microarray data, using qPCR we detected a significant increase of TNF transcript levels to 195% ($P < 0.05$) triggered by icb-1 knockdown (Fig. 4a). We then examined to what extent shRNA-triggered knockdown of icb-1 would affect the LPS-induced expression of TNF. A significant upregulation of TNF mRNA levels in MCF-7 cells was observed after treatment with LPS concentrations of 1 or 10 μg/ml. Although the effect of LPS on TNF expression

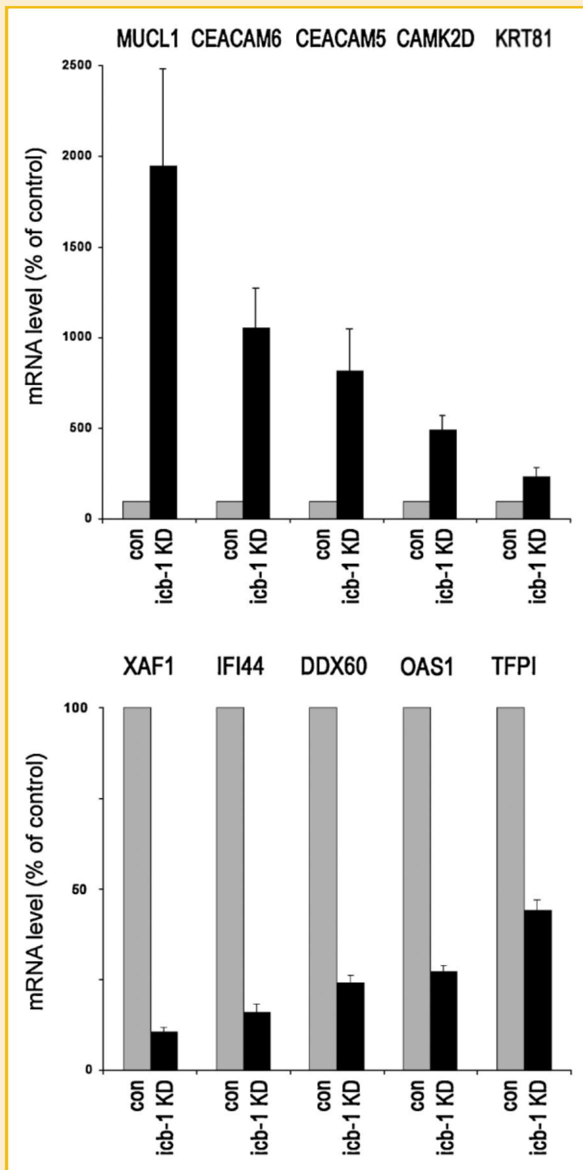

Fig. 1. Microarray data validation by qPCR analysis. Shown are the relative transcript levels of the indicated up- or downregulated genes in MCF-7 (icb-1 KD) cells compared to control-transfected cells. Data are expressed as percent of the expression in control cells (n = 2).

was lower in MCF-7 (icb-1 KD) cells, particularly at the 10 μg/ml concentration, this difference did not reach statistical significance (Fig. 4b).

We also examined whether expression of icb-1 would be regulated by TNF or LPS in human breast cancer cells. Icb-1 mRNA levels were significantly elevated after 48-h treatment with TNFα in all cell lines tested, with the most prominent, about fivefold induction present in MCF-7 cells (Fig. 5). LPS treatment (48 h) exerted only small effects on icb-1 expression in MCF-7 and T47-D cells, but increased transcript levels of this gene about fourfold in HS578T breast cancer cells (Suppl. 2).

IMPAIRED EFFECT OF TAMOXIFEN AND CHEMOTHERAPEUTICS ON MCF-7 (ICB-1 KD) CELLS

Given that DNA microarray and qPCR analysis revealed downregulation of a network of interferon responsive genes including several apoptosis-associated genes like apoptosis activator XAF1 in MCF-7 (icb-1 KD) cells, we were eager to know to what extent these expression changes would affect cellular response to antitumoral drugs with apoptotic action. Knockdown of icb-1 gene significantly impaired the apoptotic effect of tamoxifen on MCF-7 cells (Fig. 6). This effect was observed at standard concentrations of 1 μM tamoxifen and to an even larger extent at high tamoxifen concentrations of 25 μM. Furthermore, the effect of the apoptosis inducers actinomycin D (10 μM), staurosporine (1 μM), or camptothecin (2 μM) on MCF-7 cell viability and activation of caspase 7 was significantly decreased in MCF-7 (icb-1 KD) cells (Fig. 7).

DISCUSSION

Given that icb-1 expression level in leukocytes or leukemia cells is known to be more than 100-fold higher than in cancer cells of epithelial origin, previous large-scale expression profiling studies on clinical breast cancer samples using DNA microarray technology might have failed to reveal the significance of this gene in breast cancer. Actually, recent data from our lab (unpublished results) demonstrated that icb-1 expression in 66 breast cancer tissue samples most significantly correlated with macrophage marker CD68 (Spearmańs rho 0.734, $P < 0.0001$). Thus, as long as no reliable antibodies for detection of icb-1 protein in breast cancer tissue exist, which will allow discrimination between leukocyte and cancer cell expression, studies on the cell line level are the best alternative.

INDUCED NETWORK

Knockdown of icb-1 gene led to a significant induction of cancer-related genes associated with poor prognosis, invasion, and metastasis. Most of these genes are known targets of tumor necrosis factor α (TNFα), nuclear factor κ B (NFkB) or TGFβ1 signaling. Both TGFβ and TNFα are pleiotropic cytokines which are able to exert both anttumoral and cancer-promoting effects. TNFα-triggered NFkB activation, which seems to be the dominant action of this cytokine in our setting, is known to impair apoptosis signaling and to induce growth, invasion, and metastasis of tumor cells [Wang et al., 2008]. With regard to breast cancer, increased TNF expression has been reported to be associated In an expanded network including more genes exhibiting at least twofold upregulation, additionally involvement of estrogen receptor (ER) signaling was observed. Since neither expression of TGFβ- or TNF-receptor genes, their ligands nor of transcription factors NFkB, ESR1, or ESR2 was found to be elevated, the increased effect of these central network molecules on their target genes is proposed to be triggered by increased phosphorylation with increasing malignancy (García-Tuñón et al., 2006). TNFα is known to increase activity of TGFβ1, whereas the latter factor activates secretion of TNFα. With regard to TGFβ, paradoxically tumorigenesis counteracts the tumor suppressing activities of this factor, thus enabling TGFβ to stimulate cancer invasion and metastasis [Tian et al., 2011]. The genes presumably

TABLE II. Prediction of Gene Networks and Functions of icb-1 Gene Based on Our DNA Microarray Data Generated After *icb-1* Knockdown Using IPA Software (Ingenuity Systems, Stanford)

Predicted biological functions		
Name	*P*-value	# Molecules
Diseases and disorders		
Cancer	4.07E-19–3.43E-03	174
Reproductive system disease	2.31E-11–1.72E-03	116
Gastrointestinal disease	2.83E-11–2.70E-03	176
Molecular and Cellular Functions		
Cellular growth and proliferation	1.55E-11–359E-03	144
Cell death	3.27E-10–3.40E-03	103
Cellular development	6.76E-10–3.43E-03	111
Physiological system development and function		
Embryonic development	1.83E-09–2.70E-03	69
Organ development	1.83E-09–2.70E-03	62
Tissue development	1.83E-09–3.02E-03	109

Predicted gene networks	
Associated network functions	Score
Cell-to-cell signaling and interaction, cellular development, cellular proliferation	38
Antimicrobial response, inflammatory response, infectious disease	36
Cell-to-cell signaling and interaction, hematological system development and function, immune cell trafficking	32

Top canonical pathways	*P*-value	Ratio
Interferon signaling	4.76E-07	8/36 (0.222)
Activation of IRF by cytosolic pattern recognition receptors	3.97E-04	7/72 (0.097)

Predicted biological functions: Shown is the predicted function together with its *P*-value and the number of molecules included in the prediction. Predicted gene networks and pathways: Shown is a score value calculated by IPA software indicating association of icb-1 function with the indicated network functions, the *P*-value of association with the indicated canonical pathways and the ratio between analyzed and total pathway genes.

activated by increased TNF/NFkB- or TGFβ- signaling in our study are examples for the tumor promoting function of both cytokines.

The link between icb-1 function and TNF signaling predicted from our microarray data was confirmed by further qPCR experiments demonstrating upregulation of TNF expression in MCF-7 (icb-1 KD) cells and induction of icb-1 expression by TNF. Icb-1 has been recently suggested to act as a mediator between Toll-like receptor 4 (TLR4) and TNF activation in human macrophages [Peirce et al., 2010]. In MCF-7 cells, the effect of icb-1 knockdown on LPS-induced TNF expression did not reach statistical significance, an observation which might be explained by the epithelial origin of these cells. However, our data clearly suggesting icb-1 to be a TNF-responsive gene are supported by the detection of NFκB binding sites in the promoter region of this gene by transcription factor chromatin immunoprecipitation followed by tag sequencing (ChIP-seq) experiments (ENCODE Project Consortium, 2007).

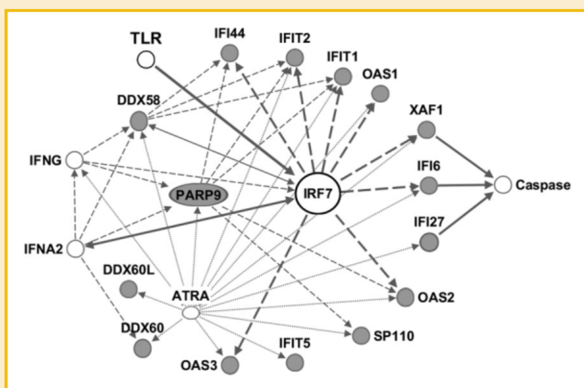

Fig. 2. Network of genes downregulated after knockdown of icb-1 in MCF-7 breast cancer cells. The majority of these interferon (IFN) response genes is known to be regulated by IFN/IRF7, PARP9, or all trans retinoic acid (ATRA). Decreased expression of XAF1, IFI6, and IFI27 is predicted to impair caspase activation. Solid arrows: Activation; dotted arrows: Regulation of expression; grey circles: More than 2.5-fold downregulated genes.

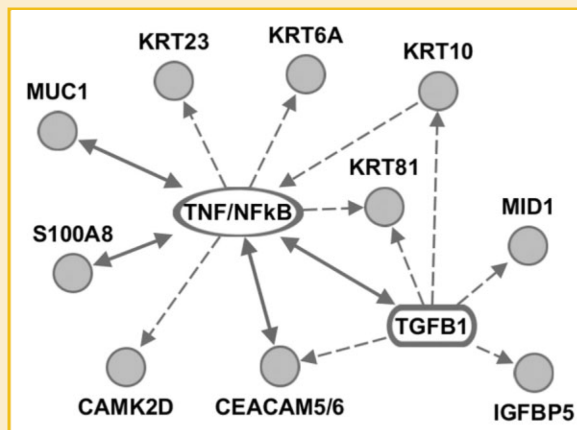

Fig. 3. Network of genes with increased expression after knockdown of icb-1 in MCF-7 cells. The majority of these genes is known to be regulated by TNFα and/or transcription factor NFκB. The second key regulator of this network is TGFβ1. Solid arrows: Activation; dotted arrows: regulation of expression; grey circles: More than 2.5-fold induced genes.

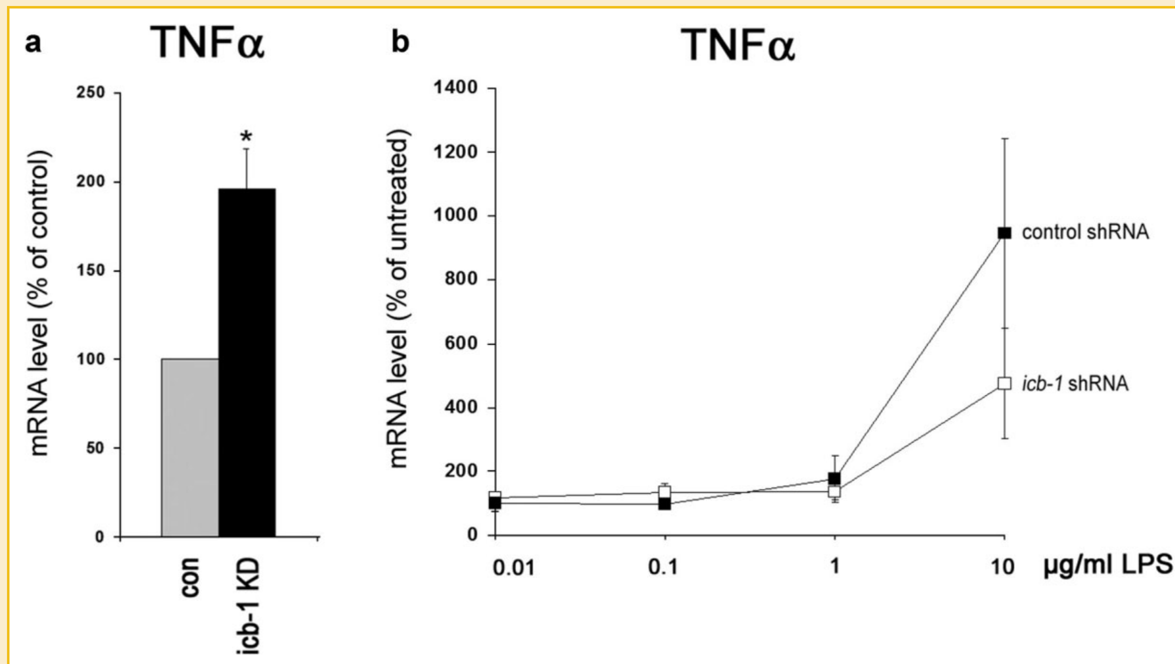

Fig. 4. Effect of an icb-1 knockdown on TNF expression. A: Expression of tumor necrosis factor (TNF) in MCF-7 (icb-1 KD) cells compared to control-transfected cells. B: LPS-triggered TNF expression subject to icb-1 knockdown. Cells were treated with the indicated concentrations of LPS for 48 h and TNF transcript levels were determined by means of RT-qPCR. (n = 3) * = $P < 0.05$ versus control.

The role of icb-1 in breast cancer cells is further elucidated by understanding the function of genes upregulated in this study. The gene exhibiting the strongest induction after icb-1 knockdown (9.68-fold) was mucin-like 1 (MUCL1), whereas mRNA levels of its better characterized relative MUC1 (mucin 1) still were 3.36-fold elevated. MUCL1, also referred to as SBEM (small breast epithelial mucin), codes for a low-weight transmembrane glycoprotein which has been shown to be overexpressed in breast cancer and to be associated with a poor prognosis (Hubé et al., 2004; Skliris et al., 2008; Ayerbes et al., 2008; Valladares-Ayerbes et al., 2009; Liu et al., 2010). Among the genes with the highest induction after icb-1

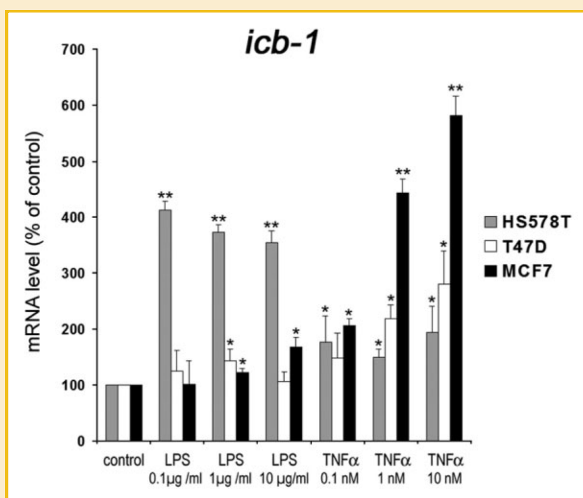

Fig. 5. Effects of LPS and TNF on icb-1 transcript levels in MCF-7 cells. Cells were treated with the indicated concentrations of LPS or TNF for 48 h and icb-1 transcript levels were determined by means of RT-qPCR. $^{*}P < 0.05$ versus control; $^{**}P < 0.01$ versus control.

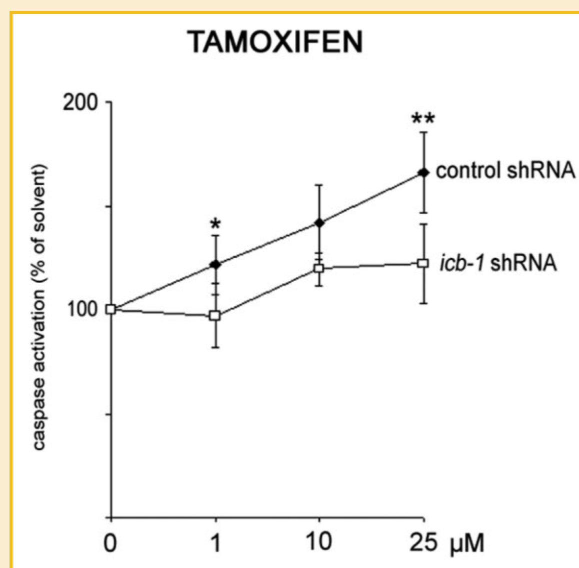

Fig. 6. Apoptotic effect of Tamoxifen on MCF-7 cells subject to icb-1 knockdown. MCF-7 (icb-1KD) and control cells were treated with the indicated concentrations of Tamoxifen for 4 h and caspase activation was determined as described in the Materials and Methods section. $^{**}P < 0.01$ versus EtOH, $^{*}P < 0.05$ versus EtOH (n = 3).

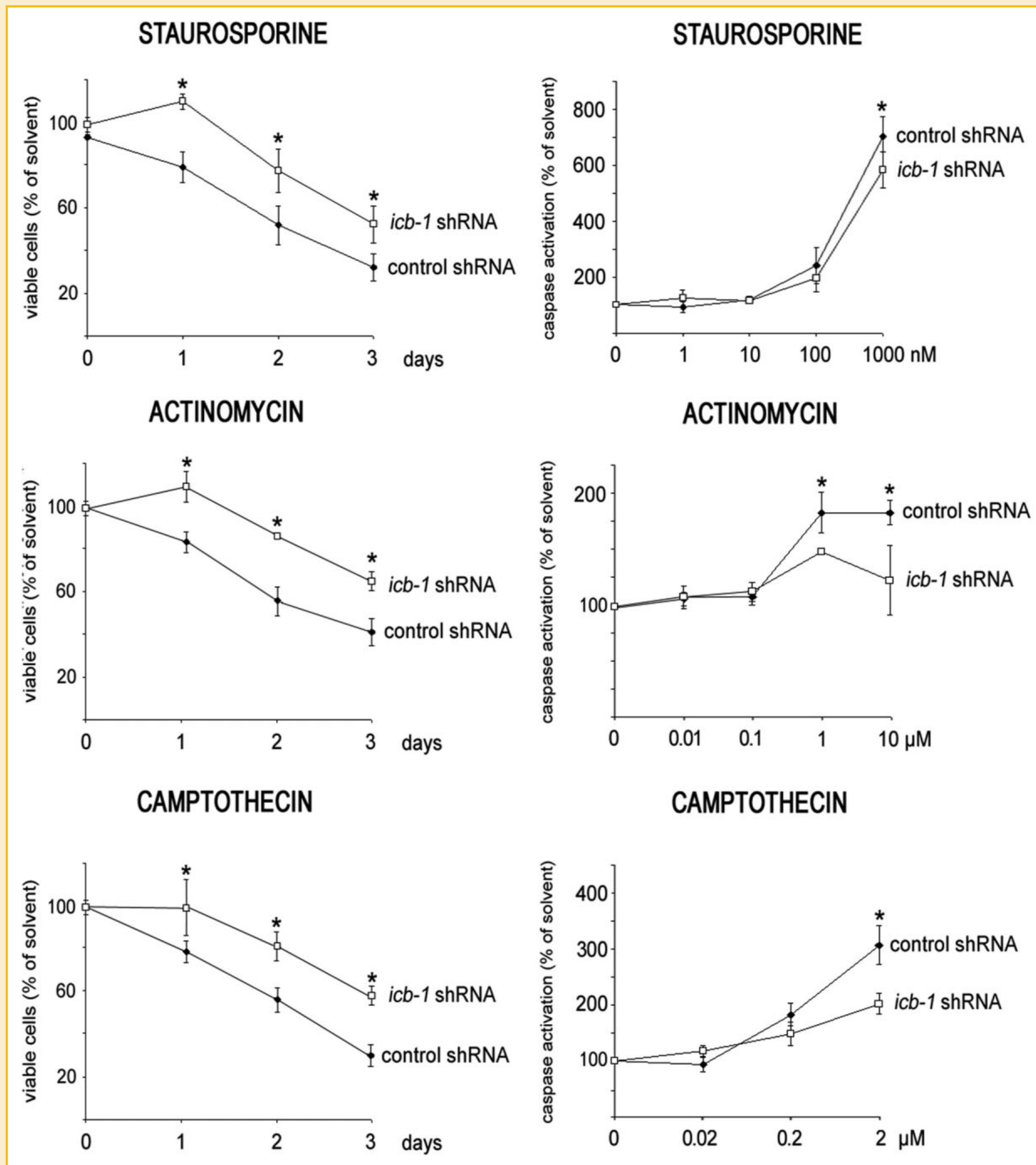

Fig. 7. Effect of apoptotic drugs on MCF-7 cells expressing icb-1 shRNA. On the left, cell viability after treatment with the indicated drugs for up to 3 days in shown. Cells were treated with 1 μM staurosporine and actinomycin D or 2 μM camptothecin. On the right, activity of caspase 7 in MCF-7 cells after 4-h treatment with the indicated concentrations is shown—data are generally expressed in percent of the vehicle control ($n=3$). $^{*}P<0.01$ versus solvent.

knockdown, several cytokeratin genes like KRT81, KRT23, KRT6, and KRT10 can be found. KRT81 (KRTHB1) is known to be upregulated by NFkB (p65/RelA), and the same is true for KRT6 [Ma et al., 1997; Gilon et al., 2008]. Both studies support presence of an increased NFkB signaling as a result of icb-1 knockdown in MCF-7 cancer cells. Increased expression of the keratin genes 6A, 10, 23, and 81 can be found in various cancer types and often is associated with a poor prognosis. [Birkenkamp-Demtroder et al., 2007; Dydensborg et al., 2009; Campayo et al., 2011; Wang et al., 2009a; Tischkowitz et al., 2007; Doljak et al., 2008; Yang et al., 2008]. Thus, downregulation of these KRT genes again links the loss of icb-1 expression to tumor progression and poor prognosis of breast cancer. The about fivefold upregulated genes carcinoembryonic antigen-related cell adhesion molecule (CEACAM) 5 and 6 [also known as cluster of differentiation (CD) 66e and CD66c] are glycoproteins which have been reported to be associated with

invasion and metastasis, and are often elevated in cancer tissue [Blumenthal et al., 2005, 2008; Lewis-Wambi et al., 2008]. Expression of CEA proteins previously has been reported to be induced by TNFα, and itself promotes production of interleukines and TNFα [Takahashi et al., 1993; Thomas et al., 2011]. The increased expression of CEACAM5 and 6 after icb-1 knockdown thus suggests that loss of icb-1 might increase the invasiveness of cancer cells. However, our microarray data did not reveal any invasion-associated gene to be upregulated in MCF-7 (icb-1 KD) cells.

DOWNREGULATED NETWORK

Knockdown of icb-1 in MCF-7 breast cancer cells also led to downregulation of a network of interferon response genes, which is predicted to result in impaired caspase activation. Particularly the decreased expression of XAF1 and IFI27, which are known activators of caspase signaling, is suggested to impair the apoptotic machinery of MCF-7 (icb-1 KD) cells [Liston et al., 2001; Rosebeck & Leaman, 2008]. X-linked inhibitor of apoptosis (XIAP)-associated factor 1 (XAF1) is a putative tumor suppressor, which has been shown to be downregulated in a number of malignancies [Liston et al., 2001; Ng et al., 2004; Lee et al., 2006; Micali et al., 2007; Qi et al., 2007; Yu et al., 2007; Sun et al., 2008; Kempkensteffen et al., 2009; Tu et al., 2009; Wang et al., 2009b]. Given that XAF1 mediates TNFα-induced apoptosis and XIAP cleavage by acting through the mitochondrial pathway [Straszewski-Chavez et al., 2007], the observed downregulation of XAF-1 triggered by icb-1 knockdown is expected to exert tumor promoting functions by increased action of apoptose inhibitor XIAP and might confer relative resistance to TNFα. Like icb-1, expression of XAF1 is induced by all-trans retinoic acid (ATRA) and interferon γ (IFNγ) [Wang et al., 2006, 2009b; Bai et al., 2008]. Thus, the strong XAF-1 downregulation we observed suggests that icb-1 gene function seems to be important for the apoptotic effects of TNFα, ATRA, and IFNγ. Confirming this conclusion, we experimentally observed the relevance of icb-1 expression for the cellular response to ATRA and IFN γ already before we were aware of the XAF1 link from the current microarray experiments [Treeck et al., 2005; Haselberger et al., 2011].

Our data showing decreased response of MCF-7 (icb-1 KD) cells to apoptotic drugs experimentally confirmed the prediction of our network analysis, that knockdown of this gene would weaken the apoptotic machinery of MCF-7 cells. Staurosporine, camptothecin, actinomycin, and tamoxifen have previously been shown to trigger apoptosis in cancer cells [Welsh, 1994; Ng et al., 2002; He et al., 2007]. The fact that MCF-7 cells are caspase-3 deficient allows identification of caspase 7 as one important caspase being involved in the apoptotic pathways affected by icb-1 knockdown [Blanc et al., 2000]. Interestingely, this is in line with the network-based prediction of XIAP activation, which is the main inhibitor of effector caspase 7.

Most of the downregulated genes were targets of transcription factor interferon regulatory factor 7 (IRF7) [Goubau et al., 2009]. IRF7 is a master regulator of IFN type 1 expression, and IRF7 expression in turn is induced by these interferons. Additional to apoptosis induction by mechanisms like XAF1 or TRAIL activation, IRF7 overexpression has been shown to inhibit proliferation of MCF-7 cells [Andrews et al., 2002; Huang et al., 2009]. Given that expression of IRF7 itself was not altered, it is tempting to speculate that icb-1 knockdown might have led to decreased IRF7 activation. This effect might be mediated by the strong decrease of DDX58 (RIG-1) expression, which is as an activator of IRF7 [Schröder & Bowie, 2005]. However, we did not observe decreased expression of type I interferons that would have been expected, what might be explained by the activity of other IRFs.

Icb-1 has been recently suggested to act as a downstream target of TLR4 [Peirce et al., 2010]. However, our data suggesting IRF7 as key molecule mediating the negative effects of icb-1 knockdown on gene expression would point at other TLRs. Whereas TLR2, 3, 7, and 9 are generally accepted activators of IRF7 via TRAF6, TLR4 has been reported to activate IRF7 under certain conditions only (Richez et al., 2009). However, if TLR4 signaling was activated by elevated levels of its ligand S100A8, and led to IRF7 activation in MCF-7 cells, icb-1 knockdown might have impaired activation of this transcription factor [Hiratsuka et al., 2008].

Besides IRF7, the gene coding for poly (ADP-ribose) polymerase family, member 9 (PARP9) seems to play an important role in this network. PARP9 is a transcription modulator being overexpressed in chemoresistant, diffuse large B-cell lymphomas, which is induced, like icb-1, by IFNγ, IFNα2, and ATRA (Csomós et al., 2010). PARP 9 is a regulator of the downregulated genes IFIT1, IFIT2, IFI44, OAS2, and SP110–thus, downregulation of PARP9 itself as observed after icb-1 knockdown might be another mechanism underlying the observed gene expression changes in this network (Juszczynski et al., 2006).

In conclusion, our data suggest that in MCF-7 breast cancer cells, icb-1 is part of an apoptosis signaling network mediating the effects of interferons and other apoptotic drugs including tamoxifen and chemotherapeutics. In this apoptotic pathway leading to caspase activation via interferon regulatory factors, icb-1 function is involved in induction of interferon response genes like tumor suppressor XAF1. On the other hand, our network analyses suggest that icb-1 gene, which itself was demonstrated to be TNF-responsive, also exerts tumor suppressor functions by limitation of TNF-triggered induction of cancer-related genes associated with poor prognosis, invasion, and metastasis.

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