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Biochemical and Biophysical Research Communications 314 (2004) 143–150
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Stat3 modulates heat shock 27 kDa protein expression in breast epithelial cells

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Received 26 November 2003

Abstract

The constitutive activation of signal transducer and activator of transcription 3 (Stat3) is frequently detected in breast carcinoma cell lines but not in normal breast epithelial cells. *Stat3* has been classified as an oncogene because activated Stat3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice. In this study, we investigated potential Stat3 regulated genes in breast cells. Upon expression of Stat3-C, a constitutively active Stat3 form, in nonmalignant telomerase immortalized breast cells (TERT), cell lysate was subjected to 2-dimensional (2-D) protein gel analysis. Our results showed that heat shock 27 kDa protein (HSP27) was markedly induced by Stat3-C expression. Further analysis demonstrated that phosphorylation of HSP27 at serine residue 78 was induced by Stat3-C in TERT breast cells as well as in MCF-10A and MDA-MB-453 breast cells. RT-PCR result confirmed that HSP27 mRNA was induced by Stat3-C in TERT cells. As the result of Stat3 knock-down by Stat3 short interfering RNA oligonucleotides in MDA-MB-468 human breast carcinoma cells, HSP27 was markedly reduced consistent with Stat3 reduction. Furthermore, we observed that Stat3 was physically associated with HSP27 and HSP90 in MDA-MB-468 breast carcinoma cells. Taken together, our findings demonstrate that constitutively activated Stat3 up-regulates HSP27 and may facilitate phosphorylation of HSP27 at serine residue 78. The up-regulation of HSP27 may be one of the underlying mechanisms with which aberrant Stat3 signaling induces cell malignancies.

Keywords: HSP27; Stat3; HSP90; Short interfering RNA; Breast carcinoma

Signal transducers and activators of transcription (STATs) are a family of transcription factors consisting of seven members: Stat1, 2, 3, 4, 5a, 5b, 6 [1–3]. Stat3 is a 90 kDa protein that plays important roles in cell differentiation and proliferation. Stat3 is activated in response to cytokines and growth factors [2,4] via upstream regulators, JAK2 and Src [5,6]. Upon activation, Stat3 is phosphorylated at tyrosine 705 and forms homo-dimer or hetero-dimer with other STAT family members [4,7]. Only dimerized Stat3 can translocate into nucleus and bind with its regulated genes [8]. Such activated Stat3 serves as a functional transcriptional stimulator and constitutive activation of Stat3 may induce cell malignancies and tumorigenesis. In various types of human cancers, aberrantly constitutive activation of Stat3 is sufficient to induce cell tumorigenesis [1,9]. A growing number of tumor-derived cell lines and samples from human cancers have been reported to contain constitutively activated STAT signaling, very frequently Stat3 and Stat5 [10–12]. The constitutive activation of Stat3 is frequently detected in breast carcinomatous cell lines but not in normal breast epithelial cells [6,12,13]. *Stat3* has been classified as an oncogene because activated Stat3 can mediate oncogenic transformation in cultured cells and tumor formation in nude mice [9,10,14].

Aberrant Stat3 signaling may participate in oncogenesis by stimulating cell proliferation, promoting angiogenesis, and conferring resistance to apoptosis induced by conventional therapies. The possible molecular mechanism of aberrant Stat3 signaling mediated oncogenesis is related to up-regulation of the potential

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doi:10.1016/j.bbrc.2003.12.048

Stat3 targeted genes involved in cell cycle control and anti-apoptosis [9,15–24]. To date, there are only a few Stat3 regulated genes that have been reported. In order to elucidate the molecular mechanism of Stat3 function in promoting cell survival or cell growth, we expressed constitutively activated Stat3 protein in telomerase immortalized non-malignant breast cells (TERT) using adenovirus-mediated Stat3-C expression system and identified the potential Stat3 regulated proteins with 2-dimensional gel (2-D gel) technology. Interestingly, heat shock protein 27 (HSP27) was found to be markedly induced in the Stat3-C expressed cells.

HSP27 is one of the small heat shock family proteins. It is also known as 24, 28 or 29 kDa protein [25,26]. In response to environmental stress, heat shock proteins are largely induced in the cells [27]. A number of reports indicate that HSP27 may have diagnostic and prognostic value for a variety of human malignancies [28] and is responsible for resistance to chemotherapy [29–34]. Besides an increase of HSP27 total protein, our further study demonstrated that phosphorylation of HSP27 at serine residue 78 was markedly induced. RT-PCR result supports that up-regulation of HSP27 by Stat3 is initiated from transcriptional level. As the result of Stat3 knock-down by Stat3 short interfering RNA (siRNA) oligonucleotides in MDA-MB-468 human breast carcinoma cells, HSP27 was markedly reduced consistent with Stat3 reduction. Together, our results suggest that *HSP27* is one of the Stat3 regulated genes. HSP27 may be involved in Stat3-mediated cell survival and oncogenesis in breast carcinoma cells. In the study of Stat3 signaling pathway, this is the first finding to base on 2-D gel analysis to identify Stat3 regulated genes. It is also the first report to demonstrate that *HSP27* is a Stat3 downstream gene.

Materials and methods

Cell lines and culture. Telomerase immortalized breast epithelial cells (TERT) and spontaneous immortalized breast cells MCF10A were cultured in F-12 medium containing 5% FBS (Invitrogen Life Technologies, Carlsbad, CA), 5 μg/ml insulin, 10 μg/ml hydrocortisone, 5 U/ml EGF (Sigma, St. Louis, MO), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen Life Technologies, Carlsbad, CA). Breast carcinoma cell lines, MDA-MB-468 and MDA-MB-453, were cultured in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. All chemical reagents were purchased from Sigma and Fisher Scientific Companies (Pittsburgh, PA) and were of analytic pure grade unless otherwise indicated.

Infection of TERT, MCF10A, MDA-MB-453 cells by Stat3-C adenovirus. Stat3-C cDNA [1,9] was cloned into an adenovirus vector, pAdCMVpLpA(-)loxP-SSP (NCV, provided by the University of Michigan Vector Core). The transcription of Stat3-C in this construct is driven by the human cytomegalovirus promoter for high-level constitutive expression. The recombinant adenovirus Stat3-C is defective in the E1 region and was propagated in human 293 cells, which provide E1A and E1B viral proteins for viral multiplication. The negative control adenovirus NCV (empty vector alone which contains the same backbone as the adenovirus Stat3-C), adenovirus anti-sense Stat3-C (The Stat3-C cDNA was inserted in reverse orientation), and adenovirus Stat3-C were purified by CsCl–ethidium bromide gradients method at the University of Michigan Vector Core. The 1.5×10^6 cells were plated into 10-cm culture plate two days prior to infection. The cells were infected with NCV, anti-sense-Stat3-C adenovirus or Stat3-C adenovirus, respectively, at a multiplicity of infection (MOI) of 50 pfu/cell. After 5 h incubation, culture medium was removed and replaced with fresh medium. The cells were continued to be cultured for another 24 h prior to harvest.

Immunoprecipitation and Western blot. The cells were lysed in RIPA buffer containing 1 mM PMSF, 1 mM protease complete inhibitor, 1 mM Na_3VO_4, and 1 mM NaF. The lysates were used at a concentration of 10 μg/μl. For immunoprecipitation, 500 μg of cell lysates from MDA-MB-468 cells was incubated with 5 μg anti-Stat3 (Cell Signaling, Beverly, MA), anti-HSP 27 (cell Signaling), and anti-HSP 90 antibodies (BD Transduction Laboratories, Lexington, KY) with gentle agitation, respectively. After 2 h, 80 μl protein A beads were added into the reaction tubes. The mixtures were incubated for another 2 h and then the beads were washed three times using RIPA lysis buffer. The proteins were resolved in 2× gel loading buffer.

For Western blot analysis, 100 μg of cell lysate was resolved on SDS–PAGE and transferred to PVDF membrane. Anti-HSP90, anti-HSP70 (BD Transduction Laboratories), anti-phospho-HSP27 (Serine78), anti-HSP27, anti-phospho-Stat3 (Tyrosine705), anti-Stat3 (Cell Signaling Inc), and anti-GAPDH (BioChemistry Lab, Temecula, CA) antibodies were used to detect corresponding proteins on the membrane, respectively.

2-D gel analysis. For 2-D gel analysis, the cells were lysed using 8 M urea containing 1 mM PMSF, 1 mM protease complete inhibitor (Roche, Mannheim, Germany), 1 mM Na_3VO_4, and 1 mM NaF. One milligram of cell lysate was applied onto p*I* 4–7 gradient gel strip (Bio-Rad, Hercules, CA) and subjected to isoelectric focusing following standard procedures. After isoelectric focusing, the gel strip was resolved on SDS–PAGE. Each sample was run 2-D gel in triplicate, independently. Compared with the controls, the differential interesting spots were excised for in-gel digestion and subjected to peptide fingerprint analysis to identify such proteins by querying protein genomic database.

RT-PCR analysis. mRNA was prepared by using Invitrogen one-step mRNA extraction kit. Primers for HSP27 mRNA; GGCAC GAGGAGCAGAGTCAGC (sense) and TGGCGGGGGAGGCA CAGC (anti-sense) were synthesized by Qiagen company (Valencia, CA). RT-PCR was performed using one-step RT-PCR kit (Invitrogen Life Technologies, Carlsbad, CA) according to manufacturer's instruction.

Stat3 knock-down by siRNA. SMARTpool of Stat3 short interfering RNA (Stat3 siRNA) oligonucleotides was purchased from Dharmacon (Lafayette, CO). Stat3 siRNA containing mixed sequences (GACCUGCAGCAAUA, GAGAAGCAUCGUGA, UUUGGUGU UUCAUA, and UCAGGUUGCUGGUC) was transfected in MDA-MB-468 cells by electroporation approach according to manufacturer's instruction. After 72 h, the cells were harvested and lysed for Western blot analysis. pEGFP-C1 vector (BD Clontech, Palo Alto, CA) was used as a positive control to determine the transfection efficiency.

Results

2-D gel analysis showed HSP27 was elevated in the Stat3-C expressed TERT cells

In order to identify the potential Stat3 regulated genes, TERT cells were infected with adenovirus Stat3-C.

As negative controls, TERT cells were also infected with NCV or adenovirus anti-sense Stat3-C. The cell lysates were resolved on 2-D gels. Then, the gel was stained with fluorescence dye to visualize peptide spots. Based on the condition of p*I* 4–7 gradient gel isoelectric focusing, most of the protein spots are identical in the un-infected cells, the cells infected with adenovirus Stat3-C, NCV, and adenovirus anti-sense Stat3-C (Fig. 1). There are several differential spots, which showed significant increase of intensity only in the cells infected with adenovirus Stat3-C (Fig. 1). These interesting spots were excised for in-gel digestion and subjected to peptide fingerprint analysis procedure. The peptide fingerprint data were submitted to protein genomic database and identify relevant proteins. The results identified two of these spots are HSP27 and HSP27 phosphorylated at serine residue 78 (Fig. 1D, indicated by arrow at 27 kDa marker level). Other protein spots increased in the same molecule weight 27 kDa region have yet to be identified. In addition, several protein spots were excised from the 90 kDa area in 2-D gel and identified as Stat3-C protein by Mass Spectrometer (Fig. 1D, indicated by arrow at 90 kDa marker level), suggesting murine Stat3 (Stat3-C) was greatly accumulated in the cells infected with adenovirus Stat3-C. HSP90 protein was not induced by Stat3-C in our study (Figs. 2 and 3). Therefore, it is the least possibility of containing HSP90. However, it may possibly contain different forms of phosphorylated Stat3-C or unidentified 90 kDa protein(s) in this 90 kDa protein area.

HSP27 was up-regulated by Stat3 in both MCF10A and MDA-MB-453 cells

To confirm the 2-D gel result of which HSP27 and phosphorylated HSP27 protein were up-regulated by Stat3-C, spontaneous immortalized breast MCF10A cells and breast carcinoma MDA-MB-453 cells were infected by adenovirus Stat3-C and subjected to Western blot analysis. MCF10A and MDA-MB-453 cells do

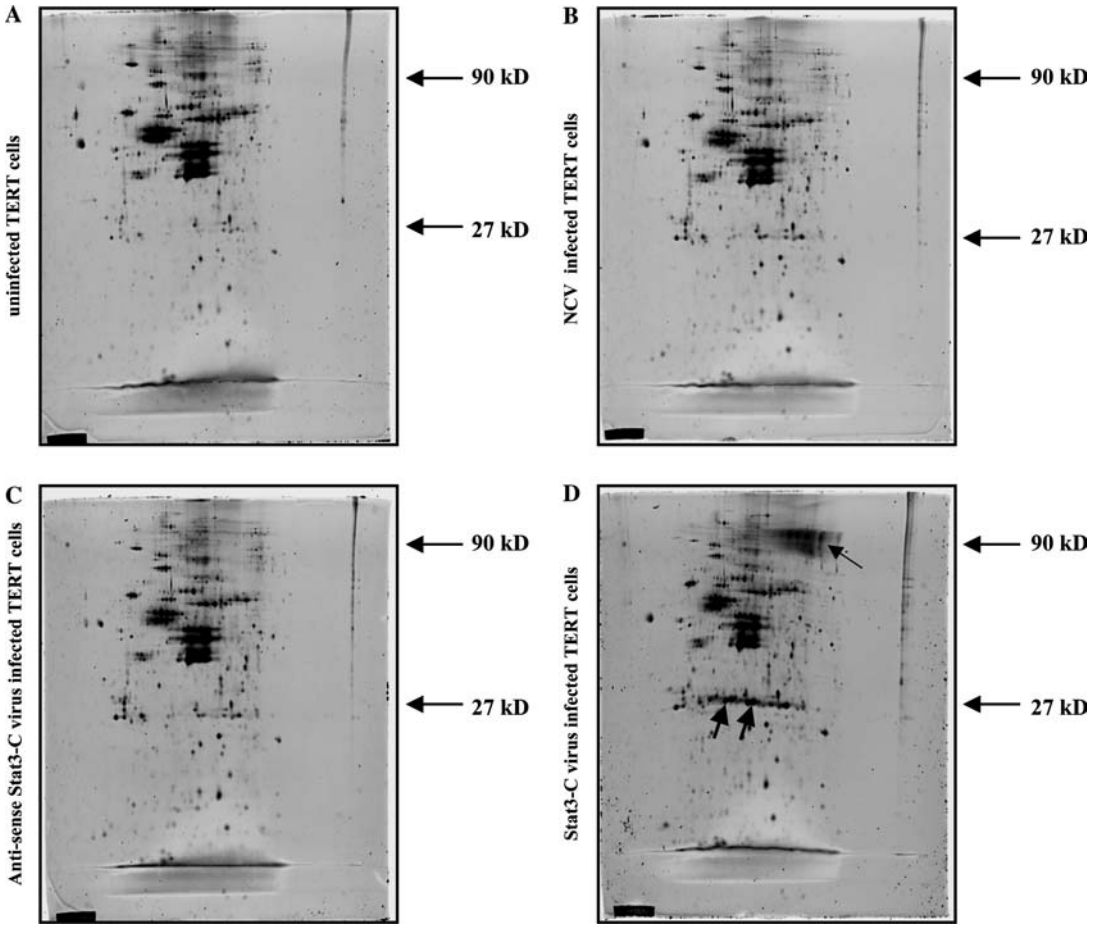

Fig. 1. Comparison of protein expression profile in TERT cells with or without expression of constitutively active of Stat3. TERT cell lysate was subjected to isoelectric focusing on p*I* 4–7 gradient gel first and then resolved on 10% SDS–PAGE. (A) Untreated TERT cells. (B) NCV infected TERT cells. (C) Adenovirus anti-sense Stat3-C infected TERT cells. (D) adenovirus Stat3-C infected TERT cells. The expression of Stat3-C (indicated by arrow at 90 kDa marker level), HSP27, and HSP27 phosphorylated at serine residue 78 (indicated by arrow at 27 kDa marker level) are showed on the 2-D gel. Each sample was run 2-D gel in triplicate, independently.

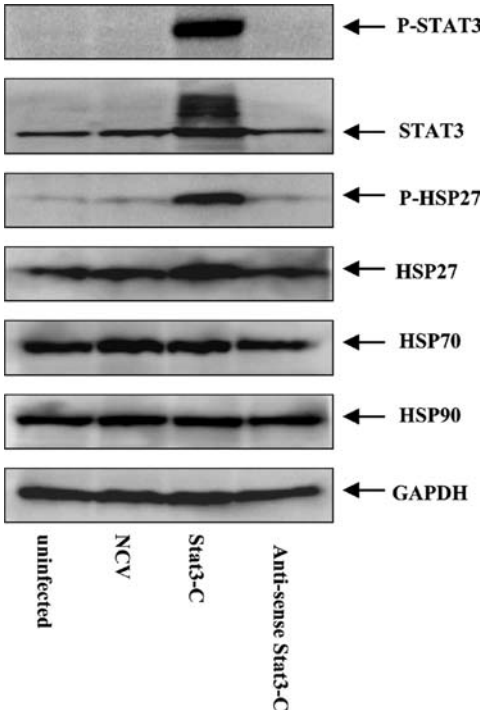

Fig. 2. Stat3 up-regulates HSP27 and HSP27 phosphorylation at serine78 in MCF10A cells. After infection by NCV, adenovirus Stat3-C or adenovirus anti-sense Stat3-C, cell lysate was resolved on 10% SDS–PAGE and subjected to Western blot analysis using anti-phospho-HSP27 (Serine78), anti-HSP27, anti-phospho-Stat3 (Tyrosine705), anti-Stat3, anti-HSP70, anti-HSP90, and anti-GAPDH antibodies, respectively.

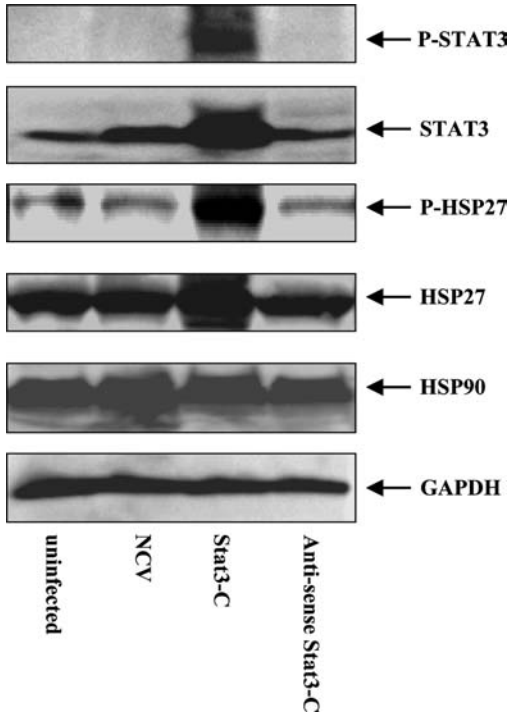

Fig. 3. Stat3 up-regulates HSP27 and HSP27 phosphorylation at Serine78 in breast carcinoma MDA-MB-453 cells. After infection by NCV, adenovirus Stat3-C or adenovirus anti-sense Stat3-C, cell lysate was resolved on 10% SDS–PAGE and subjected to Western blot analysis using anti-phospho-HSP27 (Serine78), anti-HSP27, anti-phospho-Stat3 (Tyrosine705), anti-Stat3, anti-HSP90, and anti-GAPDH antibodies, respectively.

not express constitutively active Stat3 [6,12]. In the cells infected with adenovirus Stat3-C, high levels of Stat3 phosphorylation at tyrosine residue 705 were observed (Figs. 2 and 3). Because Stat3-C was generated by substituting cysteine residues for specific amino acids within the C-terminal loop in the SH2 domain of the Stat3 molecule [9], this is a constitutively dimerizable Stat3. Therefore, Stat3 dimers were also observed in the MCF10A cells infected by adenovirus Stat3-C (Fig. 2). Protein levels of HSP27 and HSP27 phosphorylation at serine residue 78 were markedly elevated in the cells infected with adenovirus Stat3-C, parallelly. However, these findings were not observed in the cells infected with NCV and adenovirus anti-sense Stat3-C. Meanwhile, other heat shock proteins such as HSP70 and HSP90 were not induced by Stat3-C. The data are consistent with the 2-D gel result (Fig. 1).

HSP27 was up-regulated at transcriptional level

We next determined whether HSP27 was induced by Stat3-C in protein or mRNA levels. MCF10A cell mRNA extracts were subjected to RT-PCR semi-quantitative analysis. RT-PCR result showed that HSP27 mRNA was markedly elevated in MCF10A cells infected with adenovirus Stat3-C (Fig. 4). These results

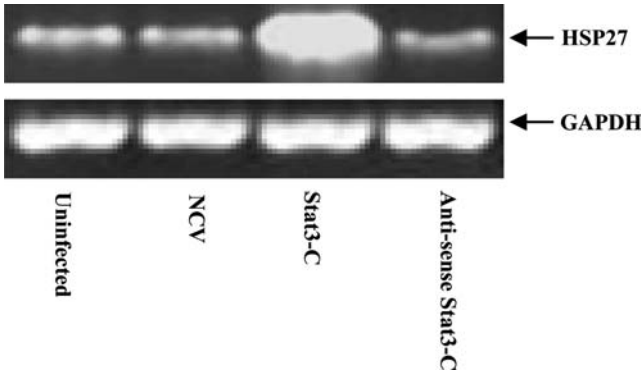

Fig. 4. RT-PCR semi-quantity result showed that Stat3 regulates HSP27 at transcriptional level. MCF10A cells were harvested after 24 h infection with NCV, adenovirus Stat3-C, or adenovirus anti-sense Stat3-C. The mRNA was extracted and subjected to RT-PCR analysis according to manufacturer's instruction.

suggest that Stat3 up-regulates HSP27 from mRNA transcriptional level.

HSP27 is involved in protein–protein interaction with Stat3

HSPs have been shown to function as molecular chaperones and form complexes with other endogenous

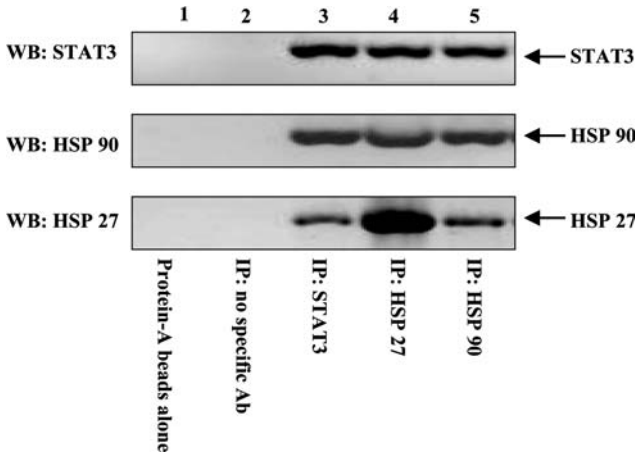

Fig. 5. HSP27 was involved in protein–protein interaction with Stat3. Breast carcinoma MDA-MB-468 cells were harvested and cell lysate was employed to immunoprecipitate HSP27, Stat3, and HSP90 using anti-HSP27, anti-Stat3 or anti-HSP90 antibodies, respectively. After resolving immuno-complexes on 10% SDS–PAGE and transfer to PVDF membrane, the membrane was probed with anti-HSP27, anti-Stat3, and anti-HSP90 antibodies, respectively. Lane 1, protein beads alone; lane 2, immunoprecipitation (IP): non-specific antibody IgG; lane 3, immunoprecipitation (IP): Stat3 and Western blot (WB): Stat3, HSP27, and HSP90; lane 4, IP: HSP27 and WB: Stat3, HSP27, and HSP90; and lane 5, IP: HSP90 and WB: Stat3, HSP27, and HSP90.

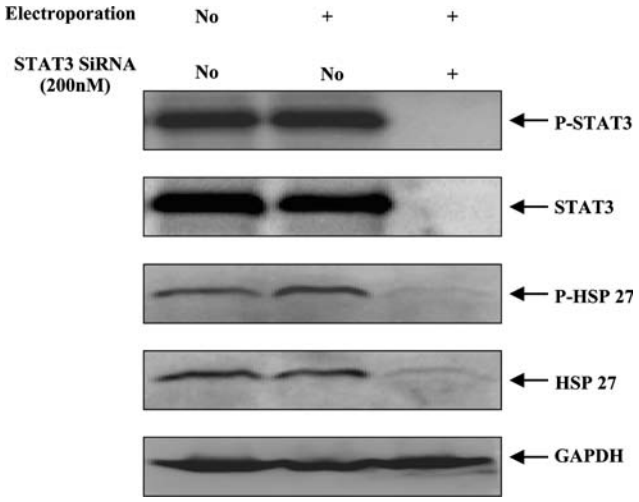

Fig. 6. Knock-down of Stat3 by siRNA. The 200 nM Stat3 siRNA SMARTpool was introduced into MDA-MB-468 breast carcinoma cells by electrical transfection. After 72 h, the cells were harvested for Western blot. Lane 1, untreated cells; lane 2, cells treated by electroporation without Stat3 siRNA; and lane 3, cells treated by electroporation with Stat3 siRNA.

proteins. To further understand the role of Stat3 in the regulation of HSPs or possible interaction with HSPs, we examined whether Stat3 may form complex with HSP27 in MDA-MB-468 breast carcinoma cells which express constitutively active Stat3 [12,35]. The Stat3 was co-immunoprecipitated with HSP27 in this cell line (Fig. 5, lane 4). As the control, neither Stat3 nor HSP27 was co-immunoprecipitated with protein A beads alone or nonspecific antibody (Fig. 5, lanes 1 and 2). Interestingly, Stat3 was also co-immunoprecipitated with HSP90 (Fig. 5, lane 5), an indicative that Stat3 is bound to HSP27 and HSP90. These results suggest that Stat3 is involved in protein–protein interaction with HSP27 and HSP90.

HSP 27 is down-regulated accompanied by the knock-down of Stat3 expression in MDA-MD-468 breast carcinoma cells

To further investigate the relationship between Stat3 and HSP27, Stat3 knock-down approach was applied in our study. Since siRNA oligonucleotides are increasingly interested in gene knock-down and cancer related studies [36–45], we introduced Stat3 siRNA SMARTpool for our investigation. Stat3 siRNA was introduced into MDA-MB-468 breast carcinoma cells. To achieve high transfection efficiency, electroporation approach was employed to introduce Stat3 siRNA into the cells. At least 90% transfection efficiency of GFP expression vector was observed in the same cell line with this approach (data not shown). After 72 h transfection, Stat3 expression and phosphorylation were significantly reduced. As a result of Stat3 knock-down, HSP27 protein expression and phosphorylation at serine 78 were markedly reduced as well (lane 3, Fig. 6). As a control, neither Stat3 nor HSP27 was affected by electrical transfection alone (lane 2, Fig. 6). This is the first report that demonstrated knock-down of Stat3 protein by siRNA.

Discussion

For studying Stat3 regulated genes, we established an adenovirus Stat3 expression system to identify its potential downstream targets. Non-malignant breast cells without Stat3 activation were employed for this study in order to examine the effect of exogenous expression of constitutively activated Stat3 on the regulation of its direct targets. This system makes it possible to identify Stat3 target genes from both mRNA transcriptional and protein translational levels. Our 2-D gel analysis results showed that in comparison to the un-infected cells, the cells infected with NCV or adenovirus anti-sense Stat3-C, the Stat3-C expressed cells showed significant increase of HSP27 and HSP27 phosphorylation at serine residue 78. The result was further confirmed in several breast cell lines including benign cell lines and malignant cell lines upon Stat3 activation. Both Stat3 and HSP27 are considered as oncoproteins and may play critical roles in cell malignancies and tumorigenesis in a variety of cancers, while HSP27 may have clinical value for prognosis and treatment, accordingly [34,46,47]. It is of interest to investigate how Stat3 could regulate HSP27

and what biological consequences could result from this regulation. Our RT-PCR semi-quantitative analysis of Stat3 mRNA induction supports that Stat3 regulates HSP27 at transcriptional level (Fig. 4). Furthermore, HSP27 expression and activity were markedly reduced accompanied by Stat3 knock-down simultaneously (Fig. 6). All data indicate that Stat3, most likely, directly regulates HSP27 expression and activity. Stat3 may directly bind to its regulated elements in HSP27 and lead to HSP27 expression change. Our data showed that HSP27 was involved in Stat3 protein–protein interaction in MDA-MB-468 breast carcinoma cells (Fig. 5). In this case, HSP27 acts as a molecular chaperon to protect Stat3 degradation from environmental proteases and phosphates.

HSPs functioning as molecular chaperones act on guiding the transport, assembly, and degradation of intracellular proteins [28]. Small HSPs (such as HSP27) are ubiquitous and are greatly conserved in amino acid sequence among all organisms [33]. These proteins can form large multimeric structures and have a wide range of cellular functions. In response to environmental stress, heat shock proteins are largely induced in the cells [25,27]. HSP27 is one of the small heat shock family proteins. An increasing number of reports also demonstrated that HSP27 might be associated with a variety of human carcinomas with high resistance to chemotherapy and poor prognosis [30,47,48]. Accordingly, HSP27 is involved in resistance or desensitization to cytochrome *c*-mediated caspase activation [49–56]. All the facts above demonstrated that HSP27 is destined to play an important role in tumor development and resistance to chemotherapy. It has been reported that heat-shock proteins as molecule chaperones form complex with many proteins. This molecule complex could stabilize such bound proteins. In our study, we observed that HSP27 physically bound with Stat3–HSP90 counterpart in breast carcinoma MDA-MB-468 cells (Fig. 5). HSP90 has been shown to complex several proteins that are involved in human oncogenesis including AKT and Her-2 [57,58]. It is possible that HSP27 may further stabilize Stat3–HSP90 complex.

HSPs appear to aid cell survival and contribute to chemo-resistance [28,31,47]. It has been reported that HSP27 could negatively regulate apoptosis through inhibition of caspase-3 activation [59,60]. In this regard, HSP27 may also be considered to be potential anticancer drug targets [33]. HSP27 alongside HSP90 may act as molecular chaperons, which protect Stat3 protein folding, conformation, dimerization, and translocation to nucleus. Human carcinomas with aberrant Stat3 signaling may induce high levels of HSP27 and become highly resistant to chemotherapy. However, it remains to elucidate how HSP27 interacts with Stat3 and whether or not it co-translocates to the nucleus with Stat3. In addition, the detailed molecular mechanisms of which HSP27 plays roles in cell malignancies deserve further elucidation. Due to both Stat3 and HSP27 proteins associated with a variety of carcinomas, it is of interest to investigate their relationship and potential roles during the development of human breast carcinoma.

Acknowledgments

We thank Dr. Bromberg (Department of Medicine, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, USA) for generously providing the Stat3-C vector. This work was supported by the NIH Grant CA096714 to J. Lin.

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