COOPERATIVITY OF THE MUC1 ONCOPROTEIN AND STAT1 PATHWAY IN POOR PROGNOSIS HUMAN BREAST CANCER

Nikolai Khodarev\textsuperscript{a,1}, Rehan Ahmad\textsuperscript{b,1}, Hasan Rajabi\textsuperscript{b}, Sean Pitroda\textsuperscript{a}, Turner Kufe\textsuperscript{b}, Cain McClary\textsuperscript{b}, Maya Datt Joshi\textsuperscript{b}, Dhara MacDermed\textsuperscript{a}, Ralph Weichselbaum\textsuperscript{a,2}, and Donald Kufe\textsuperscript{b,2}

\textsuperscript{a} Department of Radiation and Cellular Oncology, University of Chicago, Chicago, IL 60637
\textsuperscript{b} Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA 02115

Abstract

Signal transducer and activator of transcription 1 (STAT1) is activated in the inflammatory response to interferons. The MUC1 oncoprotein is overexpressed in human breast cancers. Analysis of genes differentially expressed in MUC1-transformed cells has identified a network linking MUC1 and STAT1 that is associated with cellular growth and inflammation. The results further demonstrate that the MUC1-C subunit associates with STAT1 in cells and that the MUC1-C cytoplasmic domain binds directly to the STAT1 DNA binding domain. The interaction between MUC1-C and STAT1 is inducible by IFN\textgreek{g} in non-malignant epithelial cells and constitutive in breast cancer cells. Moreover, the MUC1-STAT1 interaction contributes to the activation of STAT1 target genes, including \textit{MUC1} itself. Analysis of two independent databases demonstrated that MUC1 and STAT1 are coexpressed in about 15\% of primary human breast tumors. Coexpression of MUC1 and the STAT1 pathway was found to be significantly associated with decreased recurrence-free and overall survival. These findings indicate that (i) MUC1 and STAT1 function in an auto-inductive loop, and (ii) activation of both MUC1 and the STAT1 pathway in breast tumors confers a poor prognosis for patients.

Keywords

MUC1; STAT1; breast cancer; IFN\textgreek{g}; auto-inductive loop; inflammation

Introduction

Signal transducer and activator of transcription 1 (STAT1) is activated in the inflammatory response to type I and II interferons (IFNs). Both types of IFNs induce tyrosine phosphorylation of STAT1 by the IFN receptor-associated Janus-activated kinase (JAK) 1,
JAK2 and Tyk2 (Schindler et al., 2007). Type I IFN (IFNα, IFNβ) induces the assembly of STAT1 in the ISGF3 complex with STAT2 and Irf9. With type II IFNγ stimulation, STAT1 forms homodimers, is imported into the nucleus and activates transcription of target genes with IFNγ-activated sites (GAS; TTTCNNGAAA) (Levy and Darnell Jr., 2002; Schindler et al., 2007). IFNγ/STAT1 target genes encode proteins that in large part promote inflammation and inhibition of growth (Der et al., 1998; Schindler et al., 2007). Receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR) and the platelet-derived growth factor receptor (PDGFR), have also been shown to directly or indirectly mediate tyrosine phosphorylation and thereby activation of STAT1 (Darnell Jr. et al., 1994; Levy and Darnell Jr., 2002). In contrast to these inducible responses, STAT1 is constitutively activated in human breast and other types of tumors (Yu and Jove, 2004). The constitutive activation of STAT1 in malignant cells has been associated with the induction of prosurvival genes and suppression of the death response to ionizing radiation (IR) and other forms of stress (Khodarev et al., 2004; Khodarev et al., 2007). Based on these observations, an experimentally derived IFN-related DNA damage resistance gene signature (IRDS) that includes STAT1 and STAT1 target genes was identified in association with resistance to IR and chemotherapy (Weichselbaum et al., 2008). Analysis of a breast cancer microarray database further demonstrated that the IRDS predicts for poor outcomes for breast cancer patients receiving adjuvant chemotherapy and for local-regional control after radiation (Weichselbaum et al., 2008). These findings have supported a role for STAT1 in regulating the response of human breast cancer cells to genotoxic stress.

The mucin 1 (MUC1) oncoprotein is aberrantly overexpressed in human breast tumors (Kufe et al., 1984). MUC1 consists of two subunits that, after cleavage of a single polypeptide, form a complex of an N-terminal mucin component (MUC1-N) and a transmembrane C-terminal (MUC1-C) subunit (Ligtenberg et al., 1992; Macao et al., 2006). MUC1-C functions as a cell membrane receptor that interacts with the ligand galectin-3 and associates with EGFR (Li et al., 2001b; Ramasamy et al., 2007). The MUC1-C cytoplasmic domain (MUC1-CD) is a substrate for EGFR, c-Src, c-Abl and certain serine-threonine kinases (Li et al., 1998; Li et al., 2001a; Li et al., 2001b; Raina et al., 2006; Ren et al., 2002). MUC1-C accumulates in the cytosol of breast cancer cells and is targeted to the nucleus, where it interacts with estrogen receptor α (ERα) and coactivates ERα target genes (Leng et al., 2007; Wei et al., 2006). MUC1-C also interacts with the p53 tumor suppressor and β-catenin, and contributes to regulation of p53- and Wnt/β-catenin-mediated transcription (Huang et al., 2005; Huang et al., 2003; Wei et al., 2005). Other studies have shown that MUC1-C is targeted to mitochondria and blocks stress-induced apoptosis (Ren et al., 2004; Ren et al., 2006; Yin et al., 2007; Yin and Kufe, 2003). In concert with the involvement of MUC1-C in diverse pathways that have been linked to transformation, overexpression of MUC1-C, and specifically the MUC1-C cytoplasmic domain, in 3Y1 rat fibroblasts induces (i) an increase in growth rate, (ii) colony formation in soft agar, and (iii) tumorigenicity in nude mice (Huang et al., 2005; Li et al., 2003). MUC1-CD also induces transcriptional programs that are highly predictive of clinical outcome for patients with breast cancer (Khodarev et al., 2009; Pitroda et al., 2009). These findings have indicated that MUC1-mediated transcriptional regulation contributes at least in part to the malignant phenotype in experimental models and in breast tumors.
The present studies demonstrate that MUC1-induced transformation is linked to activation of the STAT1-induced gene network. The basis for this finding is supported by the demonstration that MUC1-C interacts directly with STAT1 and, in an autoinductive loop, MUC1 contributes to STAT1-mediated activation of the MUC1 gene itself and of STAT1 target genes. We also show that coexpression of MUC1 and STAT1 is of importance to outcome for patients with breast cancer.

**Materials & Methods**

**Cell culture**

Rat 3Y1 fibroblasts transfected to stably express an empty vector (3Y1/vector; two clones A and B) or one expressing the MUC1-C cytoplasmic domain (3Y1/MUC1-CD; two clones A and B) were grown in vitro as described (Huang et al., 2005). The 3Y1/vector and 3Y1/MUC1-CD cells were treated with 20 ng/ml rat IFN-γ (R&D Systems, Minneapolis, MN). Human ZR-75-1, ZR-75-1/vector and ZR-75-1/MUC1siRNA breast cancer cells (Ren et al., 2004) were grown in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum, 100 µg/ml streptomycin, 100 units/ml penicillin and 2 mM L-glutamine. Human MCF-10A breast epithelial cells were grown in mammary epithelial cell growth medium (MEGM; Lonza, Walkersville, MD) and treated with 20 ng/ml human IFN-γ (R&D Systems). Transfection of MCF-10A cells with siRNA pools (Dharmacon, Lafayette, CO) was performed in the presence of Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Cells were treated with the MUC1-C inhibitor GO-201 or the control CP-1 as described (Raina et al., 2009).

**Analysis of DNA microarrays**

RNA purification and hybridization with GeneChip® Rat Genome 230 2.0 Arrays (Affymetrix, Santa Clara, CA) was performed as described (Kimchi et al., 2005). The selection and analysis of genes differentially expressed in 3Y1/vector and 3Y1/MUC1-CD cells in vitro was based on previously detailed approaches (Khodarev et al., 2004; Khodarev et al., 2003). 3Y1/MUC1-CD cells were grown subcutaneously in female athymic mice and the tumors were harvested for RNA extraction for analysis of gene expression as described (Khodarev et al., 2009; Pitroda et al., 2009). Briefly, each array was hybridized with a pooled sample normalized to total RNA and consisting of RNA obtained from 3 independent xenografts or cell lines. Data were normalized using “global median normalization” across the entire dataset (Kimchi et al., 2005) and filtrated using multi-step filtration (Khodarev et al., 2004). Subsequent analysis was based on pair-wise comparisons (3Y1/vector in vitro vs. 3Y1/MUC1-CD in vitro and 3Y1/MUC1-CD in vitro vs. 3Y1/MUC1-CD in vivo) of duplicated arrays using Significance Analysis of Microarrays (SAM) (Tusher et al., 2001). Differentially-expressed probe set IDs were selected using a 2.0-fold induction cut-off level with a False Discovery Ratio set to 0. Selected probe set IDs were annotated and functionally designated using Ingenuity Pathways Analysis (IPA, Ingenuity Systems, Inc., Redwood City, CA, USA). Fisher’s exact test was used to estimate the significance of functional networks within a given experimental dataset. This method estimates the likelihood that Network Eligible Molecules from an experimental dataset are linked in a specific network by random chance. A P-value ≤0.05 is considered significant and indicates...
a non-random enrichment of an experimental dataset by members of a specific functional network.

**Immunoprecipitation and immunoblotting**

Lysates from sub-confluent cells were prepared as described (Ren et al., 2004). Soluble proteins were precipitated with anti-MUC1-C (Ab5; Lab Vision, Fremont, CA) or anti-STAT1 (Santa Cruz Biotechnology, Santa Cruz, CA). The precipitates and cell lysates were immunoblotted with anti-STAT1, anti-MUC1-C, anti-β-actin (Sigma, St. Louis, MO) and anti-IFITM1 (Proteintech Group, Chicago, IL). Immune complexes were detected with horseradish peroxidase-conjugated secondary antibodies and enhanced chemiluminescence (GE Healthcare Biosciences, Piscataway, NJ).

**In vitro binding assays**

GST, GST-MUC1-CD, GST-MUC1-CD (1–45), GST-MUC1-CD (46–72) and GST-MUC1-CD (mSRM) were prepared as described (Ahmad et al., 2007; Huang et al., 2005) and incubated with purified recombinant STAT1. GST-STAT1 and certain GST-STAT1 deletion mutants were incubated with purified MUC1-CD that had been cleaved with thrombin to remove the GST moiety. Adsorbates to glutathione-conjugated beads were analyzed by immunoblotting.

**Real-time RT-PCR**

Total RNA was isolated from cells using the RNeasy isolation kit (Qiagen, Valencia, CA). Equal amounts of RNA were analyzed with the Superscript III platinum SYBER green one step qRT-PCR kit (Invitrogen, Carlsbad, CA) on an ABI 7900HT RT-PCR machine (Applied Biosystems, Foster City, CA). The human HPRT gene was used as the internal control. The enrichment value at time zero was employed as the reference to calculate the fold-changes.

**ChIP assays**

Soluble chromatin was prepared as described (Wei et al., 2006) and precipitated with anti-STAT1, anti-MUC1-C, anti-STAT5 (Santa Cruz Biotechnology, Santa Cruz, CA) or a control non-immune IgG. The input chromatin lysate was 1% of that used for ChIP PCR (ChIP Protocol; Millipore, Billerica, MA). For re-ChIP assays, complexes from the primary ChIP were eluted with 10 mM DTT, diluted in re-ChIP buffer and reimmunoprecipitated with anti-MUC1-C. For PCR, 2 μl for a 50 μl DNA extraction was used with 25–35 cycles of amplification.

**Statistical analysis of breast cancer databases**

Two publicly available databases were analyzed that contain breast tumor expressional data from 327 (Loi et al., 2007) and 155 (Chanrion et al., 2008) patients. All statistical analyses were performed using JMP 7.1 (SAS Institute Inc. Cary, NC, USA). The raw signal intensity for each probe set ID of interest for each patient was normalized to the average value of the probe set ID across the entire database and subsequently log₂-transformed. Multiple probe set IDs for a given gene were averaged for each patient sample to obtain a representative
expression value for each gene. Expression data were clustered using hierarchical clustering via Ward’s method. To identify the genes that were differentially-expressed between the two patient clusters, F-tests were used to test the null hypothesis of equal variance for each gene between the two patient clusters. Results of the F-test (equal or unequal variance) were entered into an unpaired 2-tailed Student’s t-test to test the null hypothesis of equal magnitude of gene expression of each gene between the two patient clusters. A gene expression score for the STAT1 pathway was determined for each patient sample by calculating the average log2 level of expression across the 24-gene STAT1 pathway. STAT1 pathway expression (STAT1P+) was defined as having a gene expression score greater than zero. MUC1 expression (MUC1+) was defined as having a log2 normalized expression value greater than zero. Survival analyses were performed using proportional hazards and Kaplan-Meier statistics with log-rank tests to test the null hypothesis of no difference in survival functions between patient clusters.

Results

Linkage of MUC1 and STAT1 in a functional gene network

Stable transfection of 3Y1 cells with MUC1-CD is associated with transformation and the activation of genes that functionally contribute to tumorigenesis (Huang et al., 2005; Khodarev et al., 2009). In the present work, analysis of gene expression in 3Y1/MUC1-CD, as compared to 3Y1/vector, cells growing in vitro has identified activation of IFNγ-inducible/STAT1-dependent genes, including STAT1 itself (Fig. 1A, Supplementary Tables 1 and 2). Expression of the IFNγ/STAT1 genes was 2.15-fold higher in 3Y1/MUC1-CD, as compared to 3Y1/vector, cells (P=2.6e-3; two-tailed t-test across all genes). Moreover, growth of the 3Y1/MUC1-CD cells as tumors in nude mice, as compared to that in vitro, was associated with further activation (2.96-fold) of the IFNγ/STAT1 pathway genes (Fig. 1B, Supplementary Table 1; P=1.2e-4).

Whereas these results indicate that MUC1-CD expression is associated with activation of STAT1 signaling, we used Ingenuity Pathway Analysis (IPA) to assess linkage of the MUC1-CD and STAT1 pathways. IPA of genes differentially expressed in 3Y1/MUC1-CD cells growing in vivo identified a gene network containing MUC1 and STAT1 that is associated with cellular growth and inflammation (Fig. 2A). As measured by Fisher’s exact test, the linkage of MUC1 and STAT1 in this network was most significant of all that were analyzed at P=10e-45. This network complements the data in Fig. 1 by demonstrating that the linkage between MUC1 and STAT1 is associated with activation of IFNγ/STAT1 genes as well as a complex web of gene interactions related to growth and inflammation. For example, there appeared to be an association of both STAT1 and MUC1 with TGFβ in the gene network (Fig. 2A); however, we were unable to identify significant changes in TGFβ expression. Nonetheless, analysis of other networks linking MUC1 and STAT1 identified additional upregulated genes, including IFIT1 associated with cancer, the cell cycle and cell death (P=10e-25), IFITM1 associated with lipid metabolism, small molecule biochemistry and molecular transport (P=10e-26), IFI44L associated with the cell cycle, cancer and cell death (P=10e-24), and ISG15 associated with cancer, gastrointestinal disease, and cell growth and proliferation (P=10e-22). To further assess the effects of MUC1-CD on STAT1-
mediated transcription, we stimulated the 3Y1/vector and 3Y1/MUC1-CD cells with IFNγ (Fig. 2B). As determined by quantitative RT-PCR, expression of marker genes of the IFNγ/STAT1 pathway, including IFIT1, IFITM1, and STAT1 itself, was upregulated by MUC1-CD, confirming that MUC1-CD contributes to STAT1-mediated transcription (Fig. 2B). These results indicate that MUC1-CD-induced transformation and STAT1 pathway activation leads to the up-regulation of genes involved in diverse biological processes that contribute to tumorigenesis.

**MUC1-CD interacts directly with STAT1**

To determine whether MUC1-CD associates with STAT1, lysates from 3Y1/MUC1-CD cells were immunoprecipitated with an antibody against the MUC1 cytoplasmic domain or, as a control, with a non-immune IgG. Immunoblot analysis of the precipitates with anti-STAT1 demonstrated that MUC1-CD forms complexes with STAT1 (Fig. 3A). Incubation of GST and GST-MUC1-CD with purified recombinant STAT1 further demonstrated that MUC1-CD binds directly to STAT1 (Fig. 3B). To further define the region of MUC1-CD responsible for the interaction, we incubated MUC1-CD deletion mutants with STAT1. Binding to STAT1 was predominant with MUC1-CD (46–72), indicating that the C-terminal region of MUC1-CD confers the interaction (Fig. 3B, left). In concert with these results, mutation of the serine-rich motif (SRM: SAGNGGSSLS to AAGNGGAAAA) in the MUC1-CD C-terminal region abrogated the interaction with STAT1 (Fig. 3B, right). The structure of STAT1 includes a dimerization domain at the N-terminus, a central DNA binding domain (DBD) and C-terminal transactivation domain (Levy and Darnell Jr., 2002) (Fig. 3C). Incubation of purified MUC1-CD with STAT1 deletion mutants demonstrated binding to the DBD and not the dimerization or transactivation domains (Fig. 3C). These findings indicate that MUC1-CD associates with STAT1 in 3Y1/MUC1-CD cells through a direct interaction between these proteins.

**Interaction between MUC1-C and STAT1 is an IFNγ-inducible response in MCF-10A breast epithelial cells**

The non-malignant MCF-10A breast epithelial cells express endogenous MUC1 (Ahmad et al., 2007). As shown for human mammary epithelial cells (Lagow and Carson, 2002), stimulation of MCF-10A cells with IFNγ was associated with marked upregulation of MUC1-C protein (Fig. 4A, left). RT-PCR analysis of the IFNγ-treated MCF-10A cells further demonstrated increases in MUC1 mRNA levels, consistent with transcriptional activation (Fig. 4A, right). Notably, there was little if any association between MUC1-C and STAT1 in unstimulated MCF-10A cells; however, IFNγ stimulation was associated with the detection of MUC1-C-STAT1 complexes (Fig. 4B). To determine whether STAT1 is responsible for IFNγ-induced upregulation of MUC1 expression, the MCF-10A cells were transfected with control or STAT1 siRNA pools. As expected, induction of STAT1 expression by IFNγ was attenuated by the STAT1 siRNA (Fig. 4C). Moreover, silencing STAT1 was associated with attenuation of IFNγ-induced expression of MUC1-C (Fig. 4C). Based on the findings that MUC1-CD promotes IFNγ-induced expression of STAT1 in 3Y1 cells, MCF-10A cells were treated with the MUC1-C inhibitor, GO-201 (Raina et al., 2009), and then stimulated with IFNγ. Inhibiting MUC1-C function attenuated IFNγ-induced induction of MUC1-C expression, supporting a role for MUC1 in upregulating MUC1 gene expression. 

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activation (Fig. 4D). In addition and significantly, GO-201 treatment was associated with inhibition of IFNγ-induced expression of STAT1 (Fig. 4D). Moreover, GO-201 inhibited induction of the IFN-induced transmembrane protein 1 (IFITM1), which is encoded by an IFNγ/STAT1 pathway target gene (Deblandre et al., 1995). By contrast, an inactive form of GO-201, designated CP-1 (Raina et al., 2009), had little effect (Fig. 4D). To confirm these findings, MCF-10A cells were transfected with control or MUC1 siRNA pools as described (Ahmad et al., 2009) and then stimulated with IFNγ. Real-time RT-PCR analysis demonstrated that IFNγ-induced activation of IFITM1 gene expression is blocked by silencing MUC1 (Fig. 4E). These findings indicated that both STAT1 and MUC1 contribute to the induction of the MUC1 gene and STAT1-dependent genes in the IFNγ response.

MUC1-C-STAT1 complexes occupy the MUC1 gene promoter

To determine whether MUC1-C is detectable in the STAT1 transcription complex, ChIP analysis was performed on a consensus STAT binding motif (−503 to −495; TTCCGGGAA) in the MUC1 promoter that has been documented as a STAT1 binding site (Gaemers et al., 2001). Precipitation of chromatin from MCF-10A cells with anti-STAT1 demonstrated a low level of STAT1 on the STAT binding site (SBS) (Fig. 5A). By contrast, STAT1 occupancy was undetectable on a control region (CR; +4524 to +4745) (Fig. 5A). Significantly, treatment with IFNγ was associated with an increase in STAT1 occupancy (Fig. 5A). ChIP analysis also demonstrated that MUC1-C occupies the SBS in response to IFNγ stimulation (Fig. 5B). Overexpression of endogenous MUC1 in ZR-75-1 breast cancer cells is associated with constitutive binding of MUC1-C and STAT1 (Fig. 5C). ChIP analysis of the MUC1 promoter in ZR-75-1/vector cells further demonstrated that STAT1 constitutively occupies the SBS and not the control region (Fig. 5D). Moreover, re-ChIP studies confirmed that MUC1-C coprecipitates with STAT1 on the STAT binding site (Fig. 5D). Notably, stable silencing of MUC1 in ZR-75-1/MUC1siRNA cells (Ahmad et al., 2007) resulted in decreased STAT1 occupancy of the SBS and, as expected, loss of MUC1-C in the re-ChIP (Fig. 5D). In contrast to these results and as a control, there was no detectable STAT5 occupancy of the MUC1 promoter SBS in MCF-10A and ZR-75-1 cells (Supplemental Fig. S1). These findings demonstrate that MUC1-C associates with the STAT1 transcription complex and that MUC1-C contributes to STAT1 occupancy of the MUC1 promoter.

MUC1 associates with STAT1 pathway activation in poor prognosis human breast tumors

An expressional database representing 327 cases of breast cancer (80.1% ER+) (Loi et al., 2007) was analyzed to assess the clinical significance of MUC1 and STAT1 coexpression. We found that MUC1 and STAT1 were coexpressed in 13.8% (n=45) of the breast tumors (Fig. 6A). STAT1 only was expressed in 17.7% of the tumors and MUC1 only expression was detected in 23.9%. Hierarchical clustering of the database based on expression of the STAT1 pathway (24 genes) (Khodarev et al., 2004; Weichselbaum et al., 2008) demonstrated differences in expression throughout the 327 breast tumors (Fig. 6B). Importantly, STAT1 pathway expression was significantly (Fisher’s exact test; P=0.0003) associated with coexpression of MUC1 (Fig. 6B), indicating that MUC1 expression is associated with activation of the STAT1 pathway. Using Kaplan-Meier survival analysis, we found that patients with tumors coexpressing MUC1 (MUC1+) and the STAT1 pathway
(STAT1P+) (n=49) had significantly reduced (log-rank P=0.036) recurrence-free survival compared to patients with tumors without coexpression (n=257) (Fig. 6C). No differences in recurrence-free survival were found between MUC1−/STAT1P−, MUC1+/STAT1P−, or MUC1−/STAT1P+ patients (log-rank P>0.05). Overall survival data are not available for this database. Also, coexpression of MUC1 and STAT1 was not significantly associated with ER status (Fisher’s exact test; P=0.66). The breast cancer database (Loi et al., 2007) used for these studies does not provide information about ErbB2 expression, thus it was not possible to assess coexpression of MUC1 and STAT1 in triple-negative breast cancers. We also found that MUC1+/STAT1P+ status is not associated with presence of positive lymph nodes, high tumor grade (grade 2 and 3), or tumor size (Fisher’s exact test; 0.23 ≤ P ≤ 0.66). Given that coexpression is not linked to these tumor parameters, we hypothesized that MUC1+/STAT1P+ status could enhance the identification of clinically aggressive tumors. In this regard, for patients with high grade tumors (grades 2 and 3), MUC1+/STAT1P+ status (n=32) significantly predicted (log-rank P=0.030) reduced recurrence-free survival compared to non-coexpressors (n=169) (Fig. 6D). There were again no differences in recurrence-free survival among patients with MUC1−/STAT1P−, MUC1+/STAT1P−, or MUC1−/STAT1P+ (log-rank P>0.05) high grade tumors.

Analysis of an independent database of 155 breast cancers (94.8% ER+) (Chanrion et al., 2008) demonstrated coexpression of MUC1 and STAT1 in 16.1% (Fig. 7A). The results also confirmed that expression of the STAT1 pathway across the 155 tumors was significantly associated with MUC1 expression (Fisher’s exact test P<0.0001; Fig. 7B). Notably, patients with MUC1+/STAT1P+ tumors had a 7.38 fold-increased risk for recurrence (95% confidence interval (CI): 1.66–23.7; P=0.012) and a 4.10 fold-increased risk for death (95% CI: 1.55–9.11; P=0.0070) compared to non-coexpressors (Fig. 7C). Furthermore, as found for the first database, grade 2 and 3 tumors with MUC1+/STAT1P+ status had significantly increased risks for recurrence (Hazard Ratio (HR)=6.21; 95% CI: 1.40–19.94; P=0.020) and death (HR=3.40; 95% CI: 1.28–7.57; P=0.017) compared to non-coexpressors (Supplementary Table 3). Taken together, these data demonstrate that MUC1 and STAT1 are coexpressed in breast tumors and that activation of the STAT1 pathway in association with MUC1 expression predicts reduced recurrence-free and overall survival in patients.

Discussion

MUC1 and STAT1 form a gene network

STAT1 is constitutively activated in breast and other types of cancer, although the functional significance of this response has been largely unknown (Yu and Jove, 2004). Indeed, STAT1 target genes in the non-malignant setting have been associated with inflammation, anti-proliferative effects and a tumor suppressor function (Schindler et al., 2007). However, paradoxically, recent studies have shown that constitutive activation of the STAT1 pathway in malignant cells in vitro and in human breast tumors confers resistance to genotoxic anti-cancer agents (Khodarev et al., 2004; Khodarev et al., 2007; Weichselbaum et al., 2008). Notably, the overexpression of MUC1 in breast cancer cells confers resistance to DNA-damaging chemotherapeutic agents (Ren et al., 2004), suggesting that MUC1 and STAT1 may play similar roles. In the present work, analysis of 3Y1 cells transformed with...
MUC1-CD demonstrated upregulation of the \textit{STAT1} gene and STAT1 target genes in vitro and in an animal xenograft model. Moreover, IFN\textsubscript{\gamma}-induced activation of the STAT1 pathway was increased by MUC1-CD expression, supporting a potential role for MUC1-CD in promoting STAT1-mediated transcription. By using Ingenuity Pathway Analysis to functionally classify genes differentially expressed in MUC1-CD-transformed cells, we confirmed a highly significant interaction between the MUC1 and STAT1 pathways. This network included a set of genes encoding proteins involved in the inflammatory response, consistent with that found for the IFN\textsubscript{\gamma}/STAT1 pathway. In contrast to induction of anti-proliferative genes by the IFN\textsubscript{\gamma}/STAT1 pathway in non-malignant cells, the MUC1 and STAT1 network associated with transformation also included genes that confer a proliferative response. These findings raised the possibility that the MUC1 and STAT1 proteins interact in the regulation of STAT1-mediated gene transcription.

### MUC1-C interacts directly with STAT1 in an auto-inductive loop

The MUC1-C transmembrane subunit is targeted to the nucleus of breast cancer cells where it interacts with ER\textsubscript{\alpha} on estrogen-responsive promoters and contributes to the regulation of ER\textsubscript{\alpha}-mediated transcription (Wei \textit{et al.}, 2006). MUC1-C has also been directly implicated in the regulation of other transcription factors, including p53 (Wei \textit{et al.}, 2005; Wei \textit{et al.}, 2007). The present studies demonstrate that MUC1-C associates with STAT1 as an IFN\textsubscript{\gamma}-inducible response in MCF-10A breast epithelial cells and constitutively in ZR-75-1 breast cancer cells. This interaction is mediated, at least in large part, by direct binding of the MUC1-CD C-terminal region and the STAT1 DBD. Significantly, mutation of the MUC1-CD serine-rich motif in the C-terminal region abrogated the interaction with STAT1 and, as reported, also blocks MUC1-CD-induced anchorage-independent growth and tumorigenicity of 3Y1 cells (Huang \textit{et al.}, 2005). In addition, little is known about proteins that interact with the STAT1 DBD (Shuai, 2000). This region contains a functional nuclear export signal that interacts with CRM1 and confers leptomycin B-sensitive export of nuclear STAT1 that has undergone tyrosine dephosphorylation (McBride \textit{et al.}, 2000; Meyer \textit{et al.}, 2003). The STAT1 DBD also contains two lysines that, when acetylated by the histone acetyltransferase CBP, promotes tyrosine dephosphorylation of STAT1 by TCP45 (Krämer \textit{et al.}, 2006; Krämer \textit{et al.}, 2009). Thus, binding of MUC1-C to the STAT1 DBD could affect interactions with CBP and/or TCP45 and prolong occupancy of STAT1 on its cognate responsive elements. In this regard, ChIP analysis demonstrated that both MUC1-C and STAT1 occupy the STAT binding site in the \textit{MUC1} promoter. Moreover, silencing MUC1 was associated with decreased STAT1 occupancy of that site, indicating that MUC1-C may delay STAT1 latency. The demonstration that (i) MUC1-C promotes STAT1-mediated transcription of the \textit{MUC1} gene, and (ii) downregulation of MUC1-C levels blocks induction of STAT1 and IFITM1 expression provided further support for the induction of an auto-inductive loop in which MUC1-C and STAT1 work cooperatively to activate expression of STAT1-dependent genes, including the \textit{MUC1} gene itself (Fig. 7C). The results also demonstrate that inhibition of MUC1-C function with GO-201 blocks IFN\textsubscript{\gamma}-induced activation of the STAT1 pathway. Notably, GO-201 induces complete regressions of human breast tumor xenografts in preclinical models and thus could confer these responses, at least in part, by downregulation of the STAT1 pathway (Raina \textit{et al.}, 2009). Based on these findings, STAT1 inhibitors could be effective in downregulating MUC1-C...
expression and thereby tumorigenicity. Whether the constitutive activation of a MUC1-STAT1 loop in breast cancer cells contributes to a function of STAT1 in promoting a more aggressive phenotype, as recently reported (Weichselbaum et al., 2008), was not known, and consequently was addressed using microarray databases from breast cancer patients treated with adjuvant therapy.

Coexpression of MUC1 and the STAT1 pathway in human breast tumors confers a poor prognosis

Stimulation of non-malignant MCF-10A breast epithelial cells with IFNγ was associated with STAT1-dependent induction of MUC1 expression, formation of MUC1-C-STAT1 complexes and occupancy of these complexes on the MUC1 promoter. In contrast to this IFNγ inducible interaction, the association between MUC1-C and STAT1 and their occupancy of the MUC1 promoter is constitutive in ZR-75-1 breast cancer cells, suggesting that activation of the MUC1 and STAT1 pathways may be present in primary breast tumors. Indeed, analysis of two breast cancer databases demonstrated coexpression of MUC1 and STAT1 in about 15% of tumors. Importantly, as determined by hierarchical clustering, MUC1 expression was significantly (P ≤0.0003) associated with activation of the STAT1 pathway. The significance of this association is supported by the finding that patients with tumors coexpressing MUC1 and the STAT1 pathway had significantly increased risks for recurrence and death. Coexpression of MUC1 and the STAT1 pathway was not related to ER status, high tumor grades (2 and 3) or tumor size. However, risk of recurrence and death was significantly increased for patients with grade 2 and 3 tumors that coexpressed MUC1 and STAT1, supporting involvement of the interaction in clinically aggressive breast cancers. These findings support activation of the MUC1 and STAT1 pathways in conferring reduced recurrence-free and overall survival in breast cancer patients and suggest that MUC1-C and/or STAT1 inhibitors could be effective in treating this population. In this context, the epithelial cell barrier is exposed to diverse forms of stress, including inflammatory signals, and the MUC1-C receptor functions in transducing signals that protect epithelial cells from stress-induced death (Ren et al., 2004; Wei et al., 2005; Yin et al., 2007). Activation of MUC1 expression by a STAT1-dependent auto-inductive loop would appear to be an inducible response in non-malignant cells that is constitutively activated in certain breast cancers. Thus, what may represent a physiologic mechanism to protect the mammary epithelium from an IFNγ/STAT1-induced response could have been exploited by breast cancers to survive under adverse inflammatory conditions through a previously unrecognized STAT1 tumorigenic function.

Supplementary Material

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Acknowledgments

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Figure 1. MUC1-CD activates transcription of STAT1 and STAT1-dependent genes
A. 3Y1/vector (clones A and B) and 3Y1/MUC1-CD (clones A and B) cells growing in vitro were analyzed for expression of the indicated genes in the IFNγ/STAT1 pathway. B. 3Y1/MUC1-CD cells growing in vitro and as tumors in nude mice were analyzed for expression of the indicated IFNγ/STAT1 target genes. Values are in log2 scale and represent expression relative to the average value of the gene across samples.
Figure 2. Functional gene network analysis links MUC1 and STAT1
A. Genes differentially expressed in 3Y1/MUC1-CD cells growing in vivo as compared to in vitro were analyzed using Ingenuity Pathway Analysis to identify functional networks. The gene network containing MUC1 and STAT1 as shown is associated with genes involved in cellular growth and inflammation. Red signifies up-regulation. Green signifies down-regulation. B. 3Y1/vector (open bars) and 3Y1/MUC1-CD (solid bars) were left untreated and treated with IFN-γ for 24 h. Total RNA was analyzed by quantitative RT-PCR for expression of the indicated genes. The results are expressed as the fold-induction (mean ±SD) for IFN-γ-treated as compared to untreated cells. The differences between values
obtained for the 3Y1/vector and 3Y1/MUC1-CD cells were significant at p values ranging from 0.035 to 0.00078.
Figure 3. MUC1-C interacts directly with STAT1
A. Lysates from 3Y1/MUC1-CD cells were immunoprecipitated with anti-MUC1-C or a control IgG. The precipitates were immunoblotted with the indicated antibodies. B. Amino acid sequence of MUC1-CD and MUC1-CD (mSRM) (upper panel). GST, GST-MUC1-CD, GST-MUC1-CD (1–45) and GST-MUC1-CD (46–72) bound to glutathione beads were incubated with purified recombinant STAT1 (lower left). GST, GST-MUC1-CD and GST-MUC1-CD (mSRM) bound to glutathione beads were incubated with recombinant STAT1 (lower right). The adsorbates were immunoblotted with anti-STAT1. Input of the GST and GST-MUC1-CD proteins was assessed by Coomassie blue staining. C. Structure of STAT1

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(upper panel).GST, GST-STAT1 (full length; amino acids 1–750), GST-STAT1 (N-terminal; amino acids 1–324), GST-STAT1 (DBD; amino acids 295–491) and GST-STAT1 (C-terminal; amino acids 464–750) bound to glutathione beads were incubated with purified MUC1-CD. Adsorbates were immunoblotted with anti-MUC1-C. Input of GST and GST-STAT1 fusion proteins was assessed by Coomassie blue staining.
Figure 4. Induction of MUC1 by IFNγ in MCF-10A cells is conferred by both STAT1 and MUC1

A. MCF-10A cells were treated with IFNγ for the indicated times. Lysates were
immunoblotted with the indicated antibodies (left). MUC1 and, as a control, β-actin mRNA
levels were determined by RT-PCR (right). B. MCF-10A cells left untreated or treated with
IFNγ for 24 h were immunoprecipitated with anti-STAT1. The precipitates were
immunoblotted with the indicated antibodies. C. MCF-10A cells were transfected with
control or STAT1 siRNA pools for 24 h and then left untreated or stimulated with IFNγ for
24 h. Lysates were immunoblotted with the indicated antibodies. D. MCF-10A cells were left
untreated and treated with 5 μM GO-201 or CP-1 for 3 days and then stimulated with IFNγ
for 24 h. Whole cell lysates were immunoblotted with the indicated antibodies. NS: non-
specific band. E. MCF-10A cells were transfected with control or MUC1 siRNA pools for 72
h. The transfected cells were left untreated or stimulated with IFNγ for 24 h. Total cellular
RNA was analyzed for IFITM1 expression by real-time RT-PCR. The results are expressed
as the fold activation (mean±SD from three experiments) compared with that obtained with
cells transfected with the control siRNA and left untreated (assigned a value of 1).
Figure 5. MUC1-C associates with the STAT1 transcription complex
A. Soluble chromatin from MCF-10A cells left untreated or treated with IFNγ for 24 h was immunoprecipitated with anti-STAT1. The final DNA extractions were amplified by PCR with pairs of primers that cover the STAT binding site (SBS; −689 to −414) and the control region (CR; +4524 to +4745) in the MUC1 promoter. B. Soluble chromatin from MCF-10A cells left untreated or treated with IFNγ for 24 h was immunoprecipitated with anti-MUC1-C and analyzed for SBS and CR sequences. C. Lysates from ZR-75-1 cells were immunoprecipitated with anti-MUC1-C or a control IgG. The precipitates were immunoblotted with the indicated antibodies. D. Soluble chromatin from ZR-75-1/vector and ZR-75-1/MUC1siRNA cells was precipitated anti-STAT1 and analyzed for MUC1.
promoter SBS and CR sequences. In re-ChIP analysis, the anti-STAT1 precipitates were released, reimmunoprecipitated with anti-MUC1-C and then analyzed for MUC1 promoter sequences.
Figure 6. Coexpression of MUC1 and the STAT1 pathway is associated with reduced recurrence-free survival

A. An expression database derived from 327 breast tumors was analyzed for expression of MUC1 (MUC1+) and STAT1 (STAT1+). Coexpression was detectable in 45 tumors (13.8%). Hierarchical clustering for expression of the STAT1 pathway (24 genes) was performed for the entire database. Relative expression values are in log2 scale. Activation of the STAT1 pathway (STAT1P+) was significantly associated with MUC1 expression (49 tumors; Fisher’s exact test, P=0.0003). B. Kaplan-Meier curves for recurrence-free survival were determined for patients with MUC1+/STAT1P+ tumors (n=49; red curve) compared to those patients without coexpression (n=257; blue curve). C. Kaplan-Meier curves were determined for recurrence-free survival of patients with grade 2/3 tumors that are MUC1+/STAT1P+ (n=32; red curve) compared to those patients with grade 2/3 tumors without coexpression (n=169; blue curve).
Figure 7. STAT1 pathway activation linked to MUC1 expression is associated with increased risk of death

A. An additional expressional database derived from 155 breast tumors was analyzed for expression of MUC1 (MUC1+) and STAT1 (STAT1+). Coexpression was detectable in 25 tumors (16.1%). Hierarchical clustering for expression of the STAT1 pathway (24 genes) was performed for the MUC1+ tumors. Relative expression values are in log2 scale. Activation of the STAT1 pathway (STAT1P+) was significantly associated with MUC1 expression (P<0.0001). Genes having significantly increased expression in MUC1+ tumors are shown. B. Hazard ratios for recurrence-free and overall survival were determined for patients with MUC1+/STAT1P+ tumors (n=8) compared to those patients without
coexpression (n=147) (left panel). Hazard ratios for recurrence-free and overall survival were determined for patients with grade 2/3 tumors that are MUC1+/STAT1P+ (n=8) compared to those patients with grade 2/3 tumors without coexpression (n=126) (right panel). C. Proposed schema for a MUC1-STAT1 autoinductive loop.
| Gene Symbol | Vector-A in vitro (log2) | Vector-B in vitro (log2) | MUC1-CD-A in vitro (log2) | MUC1-CD-B in vitro (log2) | MUC1-CD/Vector in vitro |
|-------------|--------------------------|--------------------------|---------------------------|---------------------------|--------------------------|
| BST2        | 0.738                    | -0.235                   | -0.469                    | -0.396                    | 0.623                    |
| CCNA1       | 0.499                    | -0.339                   | -0.062                    | -0.254                    | 0.848                    |
| CXCL10      | 0.400                    | 0.500                    | -0.634                    | -0.684                    | 0.464                    |
| FLJ20035    | -2.657                   | -2.089                   | 0.773                     | 0.924                     | 9.327                    |
| GIP2        | -0.488                   | 0.168                    | 0.193                     | 0.029                     | 1.207                    |
| HERC6       | -0.825                   | -1.057                   | 0.486                     | 0.636                     | 2.833                    |
| IFI127      | 0.029                    | -1.119                   | 0.307                     | 0.358                     | 1.837                    |
| IFI35       | 0.824                    | -0.038                   | -0.701                    | -0.643                    | 0.478                    |
| IFI44       | -0.786                   | 0.230                    | 0.187                     | 0.149                     | 1.362                    |
| IFI44L      | 0.079                    | 0.094                    | 0.030                     | -0.226                    | 0.880                    |
| IFT1        | -0.632                   | 0.310                    | 0.227                     | -0.082                    | 1.176                    |
| IFT4        | -1.878                   | -2.108                   | 0.663                     | 0.936                     | 6.925                    |
| IFTM1       | -0.602                   | 0.119                    | 0.302                     | 0.033                     | 1.328                    |
| IRF7        | -0.123                   | -0.155                   | 0.163                     | 0.089                     | 1.202                    |
| LAMP3       | -0.100                   | 0.415                    | -0.318                    | -0.102                    | 0.775                    |
| MCL1        | -0.277                   | -0.053                   | 0.041                     | 0.241                     | 1.236                    |
| MDA5        | -0.070                   | -2.818                   | 0.254                     | 0.777                     | 3.889                    |
| MX1         | -1.247                   | -0.656                   | 0.288                     | 0.785                     | 2.805                    |
| OAS1        | -0.544                   | -0.818                   | 0.496                     | 0.419                     | 2.202                    |
| PLSCR1      | -0.810                   | -0.721                   | 0.492                     | 0.502                     | 2.399                    |
| STAT1       | -0.081                   | -0.312                   | 0.144                     | 0.110                     | 1.251                    |
| Gene Symbol | MUC1-CD-A in vitro (log2) | MUC1-CD-B in vitro (log2) | MUC1-CD-A in vivo (log2) | MUC1-CD-B in vivo (log2) | MUC1-CD in vitro/MUC1-CD in vivo |
|-------------|--------------------------|--------------------------|--------------------------|--------------------------|---------------------------------|
| BST2        | −0.798                   | −0.725                   | 0.477                    | 0.514                    | 2.390                           |
| CCNA1       | −0.256                   | −0.448                   | 0.445                    | 0.095                    | 1.539                           |
| CXCL10      | 0.065                    | 0.015                    | −0.062                   | −0.021                   | 0.945                           |
| FLJ20035    | −1.049                   | −0.897                   | 0.449                    | 0.691                    | 2.914                           |
| GIP2        | −0.680                   | −0.844                   | 0.500                    | 0.490                    | 2.391                           |
| HERC6       | −0.390                   | −0.240                   | 0.341                    | 0.168                    | 1.484                           |
| IFI27       | 0.016                    | 0.067                    | −0.081                   | −0.006                   | 0.942                           |
| IFI135      | −0.056                   | 0.001                    | 0.035                    | 0.018                    | 1.038                           |
| IFI44       | −0.520                   | −0.558                   | 0.404                    | 0.379                    | 1.906                           |
| IFI44L      | −1.692                   | −1.948                   | 0.781                    | 0.777                    | 6.058                           |
| IFIT1       | −0.282                   | −0.591                   | 0.350                    | 0.309                    | 1.700                           |
| IFIT4       | −1.243                   | −0.970                   | 0.471                    | 0.749                    | 3.286                           |
| IFITM1      | −3.525                   | −3.794                   | 1.008                    | 0.872                    | 24.242                          |
| IRF7        | −0.515                   | −0.589                   | 0.371                    | 0.425                    | 1.931                           |
| LAMP3       | −0.294                   | −0.078                   | 0.173                    | 0.151                    | 1.273                           |
| MCL1        | 0.110                    | 0.310                    | −0.319                   | −0.186                   | 0.726                           |
| MDA5        | −0.570                   | −0.047                   | 0.235                    | 0.241                    | 1.460                           |
| MX1         | −1.036                   | −0.539                   | 0.530                    | 0.464                    | 2.436                           |
| OAS1        | −0.091                   | −0.168                   | −0.002                   | 0.230                    | 1.184                           |
| PLSCR1      | 0.193                    | 0.203                    | −0.270                   | −0.190                   | 0.743                           |
| STAT1       | −0.366                   | −0.400                   | 0.241                    | 0.335                    | 1.592                           |
Table 3
Cox proportional hazard analysis of recurrence and death for MUC1-STAT1 pathway co-expression

| Cohort                          | HR    | Recurrence-free survival 95% CI | P    | HR    | Overall survival 95% CI | P    |
|---------------------------------|-------|---------------------------------|------|-------|-------------------------|------|
| Entire database (n=155)         | 7.38  | 1.66 – 23.7                     | 0.012| 4.1   | 1.55 – 9.11             | 0.007|
| Grade 2/3 tumors (n=134)        | 6.21  | 1.40 – 19.94                    | 0.02 | 3.4   | 1.28 – 7.57             | 0.017|

Hazard ratio, HR; Confidence interval, CI; P-value, P