Targeting MUC1-C Inhibits TWIST1 Signaling in Triple-Negative Breast Cancer

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Abstract

The oncogenic MUC1-C protein and the TWIST1 epithelial–mesenchymal transition transcription factor (EMT-TF) are aberrantly expressed in triple-negative breast cancer (TNBC) cells. However, there is no known association between MUC1-C and TWIST1 in TNBC or other cancer cells. Here, we show that MUC1-C activates STAT3, and that MUC1-C and pSTAT3 drive induction of the TWIST1 gene. In turn, MUC1-C binds directly to TWIST1, and MUC1-C/TWIST1 complexes activate MUC1-C expression in an autoinductive circuit. The functional significance of the MUC1-C/TWIST1 circuit is supported by the demonstration that this pathway is sufficient for driving (i) the EMT-TFs, ZEB1 and SNAIL, (ii) multiple genes in the EMT program as determined by RNA-seq, and (iii) the capacity for cell invasion. We also demonstrate that the MUC1-C/TWIST1 circuit drives (i) expression of the stem cell markers SOX2, BMI1, ALDH1, and CD44, (ii) self-renewal capacity, and (iii) tumorigenicity. In concert with these results, we show that MUC1-C and TWIST1 also drive EMT and stemness in association with acquired paclitaxel (PTX) resistance. Of potential therapeutic importance, targeting MUC1-C and thereby TWIST1 reverses the PTX refractory phenotype as evidenced by synergistic activity with PTX against drug-resistant cells. These findings uncover a master role for MUC1-C in driving the induction of TWIST1, EMT, stemness, and drug resistance, and support MUC1-C as a highly attractive target for inhibiting TNBC plasticity and progression.

Introduction

The epithelial–mesenchymal transition (EMT) is a program in which epithelial cells undergo remodeling of cell–cell junctions, loss of apical-basal polarity and the acquisition of migratory capabilities (1, 2). EMT is essential for embryonic tissue development (2) and the reprogramming of pluripotent stem cells (3). EMT is also involved in pathologic processes, such as wound healing and cancer (2, 4). EMT has been linked to cancer progression, the cancer stem cell (CSC) state and resistance to treatment (2, 5, 6). EMT and the reverse process of mesenchymal–epithelial transition (MET) are driven in part by EMT transcription factors (EMT-TFs), miRNAs and epigenetic regulatory mechanisms in development and cancer (7, 8). EMT-TFs, such as TWIST1, ZEB1, and SNAIL, are expressed and activated by autocrine and paracrine signaling pathways (7–9). TWIST1 is a regulator of embryonic morphogenesis that, when expressed in mammary cells, induces loss of cell–cell adhesion in association with EMT and a metastatic phenotype (10, 11). TWIST1 and other EMT-TFs regulate EMT, MET and intermediate states harboring epithelial and mesenchymal features by orchestrating gene expression patterns in concert with effectors of epigenetic reprogramming (5, 7). In this way, EMT-TFs have the capacity to control cellular plasticity by directing stable and reversible regulation of genes that dictate the epithelial and mesenchymal programs. EMT-TF–induced plasticity has been linked to cancer progression and dedifferentiation by promoting tumor heterogeneity, the CSC state and therapy resistance (5).

Accumulating evidence has supported involvement of the oncogenic MUC1-C protein in the epigenetic reprogramming of cancer cells (12). MUC1-C is positioned at the apical borders of normal epithelial cells, where it is poised to respond to stress signals, such as those associated with inflammation and damage (13). MUC1-C contributes to loss of epithelial cell polarity by downregulating Crumbs complex components (CRB3, PATJ), HUGL2 (14) and the adherens junction effector E-cadherin (15). In association with driving loss of polarity, MUC1-C is expressed at increased levels over the entire surface of carcinoma cells, where it interacts with receptor tyrosine kinases (RTK), such as EGFR and HER2, which are normally positioned at basolateral borders, and promotes their activation (13, 16). MUC1-C is also targeted to the nucleus, where it directly interacts with proinflammatory TFs, including NF-κB p65 and STAT3, and contributes to

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the regulation of their target genes (12). A mechanistic role for MUC1-C in gene regulation was advanced by the demonstration that MUC1-C activates the Polycomb Repressive Complex 1 (PRC1) and BMI1-directed ubiquitylation of H2A with depression of homeobox genes (17). MUC1-C also activates PRC2, interacts with EZH2 and drives increases in H3K27 trimethylation (H3K27me3) on promoters of the CDH1 and BRCA1 TSGs (18). In addition, MUC1-C induces the DNA methyltransferases (DNMT) 1 and 3b with increases in promoter-specific DNA methylation patterns that further contribute to repression of TSGs (19). These findings have collectively supported a role for MUC1-C in inducing epigenetic modifications that contribute to progression of the cancer cell (12).

TWIST1 is expressed at low to undetectable levels in ER-positive breast cancer cells and is upregulated in TNBC cells (20). In addition, TWIST1 expression is associated with decreases in TNBC disease-free and overall survival (21). MUC1 is also expressed in TNBC; however, there is no known relationship between MUC1 and TWIST1. The present studies support an autocrine function for MUC1-C in activating STAT3 and inducing expression of the TWIST1 EMT-TF in TNBC cells. We show that MUC1-C binds to TWIST1, forms an autoregulatory loop with TWIST1 and functions as a master regulator of EMT, stemness, and drug resistance. These findings highlight the importance of targeting MUC1-C as a potential therapeutic approach for suppressing phenotypic plasticity of TNBCs.

Materials and Methods

Cell culture

Human BT-549 basal B TNBC cells (ATCC) were cultured in RPMI-1640 medium (Corning) containing 10% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin and 10 μg/mL insulin. SUM149 basal B TNBC cells (22) were grown in Ham's F-12 medium (Corning) supplemented with 10 mmol/L HEPES, 5% FBS, 100 μg/mL streptomycin, 100 U/mL penicillin, 5 μg/mL insulin and 1 μg/mL hydrocortisone. Cells were treated with the STAT3 inhibitor S3I-201 (Sigma), the MUC1-C inhibitor GO-203, which has the D-amino acid sequence [R]9-CQCRRKN (23), and paclitaxel (PTX; Selleckchem). PTX-resistant cells were generated by continuous exposure to increasing PTX concentrations over 5 to 6 months. Cell number and viability were assessed by trypsin blue exclusion. Authentication of the cells was performed by short tandem repeat (STR) analysis. Cells were monitored for mycoplasma contamination using the MycoAlert Mycoplasma Detection Kit (Lonza).

Tetracycline-inducible and stable gene silencing and overexpression

MUC1shRNA (MISSION shRNA TRCN0000122938; Sigma) or a control scrambled shRNA (CshRNA; Sigma) was inserted into the pLKO-tet-puro vector (Plasmid #21915; Addgene). MUC1-C (AQA) cDNA was inserted into the pinducer20 vector (Plasmid #44012; Addgene). The CshRNA, MUC1shRNA, NF-kB p65shRNA (MISSION shRNA TRCN0000014685; Sigma), STAT3shRNA (MISSION shRNA TRCN0000020543; Sigma) and TWIST1shRNA (MISSION shRNA TRCN0000329811; Sigma) vectors were produced in HEK293T cells as described previously (18). Cells transduced with the vectors were selected for growth in 1–3 μg/mL puromycin or 100 μg/mL geneticin.

Real-time quantitative reverse-transcription PCR (qRT-PCR)

Total RNA was isolated using TRizol reagent (Invitrogen). cDNAs were generated using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems; ref. 18). Samples were amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and the CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories). Primers used for qRT-PCR analysis are listed in Supplementary Table S1.

Immunoblot analysis

Whole-cell and nuclear lysates were prepared in RIPA buffer containing protease inhibitor cocktail (Thermo Scientific). Immunoblotting was performed with anti–MUC1-C (HM-1630-P1ABX; Thermo Fisher Scientific), anti-TWIST1 (ab50887), anti-NF-κB p65 (ab16502), anti-ALDH1 (ab134188; Abcam), anti-pSTAT3 (#9145), anti-STAT3 (#9139), anti-ZEB1 (#3396), anti-SNAIL (#3879), anti-SOX2 (#3579), anti-ABCBl (#13342), anti-N-cadherin (#131165), anti-BMI1 (#6964P), anti-CD44 (#56405), anti-GAPDH (#5174S; Cell Signaling Technology), and anti-β-actin (#A5441; Sigma).

Chromatin immunoprecipitation assays

Soluble chromatin was precipitated with anti-MUC1-C, antiSTAT3, anti-TWIST1 or a control non-immune IgG (Santa Cruz Biotechnology). The precipitates were analyzed by qPCR using the Power SYBR Green PCR Master Mix and the ABI Prism 7300 sequence detector (Applied Biosystems). Data are reported as fold enrichment relative to the IgG control (19). Primers used for chromatin immunoprecipitation (ChIP) qPCR are listed in Supplementary Table S2.

RNA-seq and bioinformatics analysis

Total RNA was isolated from cells cultured in triplicates using TRizol reagent (Invitrogen). TruSeq Stranded mRNA (Illumina) was used for library preparation. Data were analyzed using the Hallmark Gene Set Molecular Signatures Database (24) and Metacore. The accession number for the RNA-seq data reported in this paper is GEO ACCESSION GSE134147.

Colony formation assays

Cells were seeded at 3,000 per well in 6-well plates, treated with 500 ng/mL DOX for 7 days and then stained with 0.5% crystal violet in 25% methanol. Colonies were counted under a microscope.

Invasion assays

Cell invasion assays were performed in Transwell chambers precoated with Matrigel as described previously (15).

Mammosphere assays

Single-cell suspensions were cultured in MammoCult Human Medium Kit (Stemcell Technologies) at a density of 5,000 cells per well of a 6-well ultralow attachment culture plate (Corning) and treated with DMSO or 50 ng/mL DOX (Sigma) for 7 days. Mammospheres with a diameter ≥50 μm were counted under a microscope.

Mouse tumor model studies

Six-week-old female nude mice (Taconic Farms) were injected subcutaneously in the flank with 3 × 10^6 SUM149/tet-MUC1shRNA cells in 50% Matrigel. When tumor size reached...
approximately 125 mm$^3$, the mice were pair-matched and fed without or with DOX (625 ppm, daily). Tumor measurements were recorded twice each week. Mice were sacrificed when tumors reached >1,000 mm$^3$ as calculated by the formula: \( \text{width}^2 \times \text{length}/2 \). These experiments were conducted in accordance with and approved by the Dana-Farber Cancer Institute Animal Care and Use Committee (IACUC) under protocol 03-029.

**Immunofluorescence (IF) microscopy**

Cells were fixed in 4% paraformaldehyde, incubated with 0.3% Triton X-100 and blocked with 3% BSA. The fixed cells were incubated with anti–MUC1-C or anti-TWIST1 at 4°C and then, after washing, with goat anti-anti-Armenian hamster IgG H&L labeled with Alexa Fluor 488 (Abcam) and anti-mouse IgG H&L labeled with Alexa Fluor 647 (Abcam) at room temperature. DAPI (Thermo Fisher Scientific) was used for staining of nuclei.

**Cell viability and combination index**

Cells were seeded at a density of 1,000 to 2,000 cells per well in 96-well plates. After 24 hours, the cells were treated with different concentrations of GO-203 and PTX alone and in combination. Cell viability was assessed by Alamar blue staining (Invitrogen) and the Cell Counting Kit (DOJINDO, Kumamoto, Japan) of 6 replicates. The IC$_{50}$ value was determined by non-linear regression of the dose–response data using Prism 7.0 (GraphPad Software). Drug interaction and dose–effect relationships were analyzed as described previously (25). The combination index (CI) was calculated to assess synergism (CI<1) or antagonism (CI>1).

**Statistical analysis**

Each experiment was repeated at least three times. Data are expressed as the mean ± SD. The unpaired Student t test was used to assess differences between means of two groups. A P value of <0.05 was considered a statistically significant difference.

**Results**

**MUC1-C induces TWIST1 expression in TNBC cells**

MUC1-C and TWIST1, which are constitutively expressed in TNBC cells, have been linked to the induction of EMT; however, there is no reported relationship between these proteins (15, 26). Accordingly, BT-549 cells expressing a tet-inducible control shRNA or MUC1-CshRNA were treated with DOX and analyzed for TWIST1 expression. We found that downregulation of MUC1-C (Fig. 1A) is significantly associated with decreases in TWIST1 mRNA levels (Fig. 1B). Similar results were obtained in DOX-treated SUM149/tet-CshRNA and SUM149/tet-MUC1shRNA cells (Fig. 1C and D). TWIST1 is activated by NF-kB, STAT3 and other TFs (27). MUC1-C activates the ZEB1 EMT-TF by NF-kB p65-mediated signaling (15). However, silencing NF-kB p65 had no apparent effect on TWIST1 expression, supporting a distinct regulatory mechanism (Supplementary Fig. S1A and S1B). Studies in BC cells have shown that the MUC1-C cytoplasmic domain binds directly to JAK1 and STAT3, and in turn drives JAK1-mediated STAT3 phosphorylation (28). Silencing MUC1-C in the TNBC cells had no detectable effect on STAT3 mRNA levels (Supplementary Fig. S2A and S2B). Instead, we found that silencing MUC1-C in BT-549 (Fig. 1E) and SUM149 (Fig. 1F) cells results in suppression of pSTAT3 and TWIST1. In support of these findings, targeting STAT3 genetically (Supplementary Fig. S3A and S3B) or with the S3I-201 inhibitor (Supplementary Fig. S3C) decreased TWIST1 expression, suggesting that MUC1-C induces TWIST1 in TNBC cells by a STAT3-mediated mechanism.

**MUC1-C is necessary for driving TWIST1 activation**

The MUC1-C cytoplasmic domain contains a CQC motif that is necessary for MUC1-C homodimerization, nuclear import and oncogenic function (Fig. 2A; ref. 16). Treatment of BT-549 and SUM149 cells with the cell-penetrating GO-203 peptide, which targets the CQC motif, decreased pSTAT3 and TWIST1 levels (Fig. 2B). In addition, DOX-induced overexpression of tet-MUC1-C with a CQC-AQA mutation, which functions as a dominant-negative for MUC1-C function (29), was associated with suppression of pSTAT3 and downregulation of TWIST1 (Fig. 2C), further supporting the notion that MUC1-C activates pSTAT3 and thereby TWIST1 expression. The TWIST1 promoter contains three potential STAT3 binding motifs in the region from positions –60 to –162 upstream to the transcription start site (ref. 27; Fig. 2D). In this respect, we found that MUC1-C forms a nuclear complex with STAT3 in BT-549 and SUM149 cells (Supplementary Fig. S4A and S4B). ChIP studies further demonstrated that MUC1-C and STAT3 occupy this region (Fig. 2E) and, importantly, that silencing MUC1-C decreases STAT3 occupancy (Fig. 2F). Targeting MUC1-C with DOX-induced silencing or GO-203 treatment was also associated with downregulation of TWIST1 promoter activation as assessed using a pTWIST1-Luc reporter (Fig. 2G and H), demonstrating that MUC1-C is necessary for driving TWIST1 activation.

**MUC1-C and TWIST1 form an autoregulatory circuit**

The MUC1-C cytoplasmic domain is an intrinsically disordered structure that has the capacity to interact with multiple TFs, including NF-kB, ZEB1 and STAT3 (15, 28, 30, 31). Accordingly, commounprecipitation studies were performed to determine whether MUC1-C also associates with TWIST1. In this way, we found that MUC1-C/TWIST1 complexes are detectable in the nucleus (Fig. 3A). In assessing whether the interaction is direct, we found that GST–MUC1-CD binds to purified recombinant TWIST1 in vitro and that the CQC motif is dispensable for binding (Fig. 3B). Experiments with MUC1-CD deletion fragments further demonstrated that MUC1-CD(46-72), and not MUC1-CD(1-45), confers the interaction (Fig. 3C). Of interest in this regard, we found that the MUC1 promoter contains two consensus TWIST1 binding motifs (Fig. 3D). ChIP assays performed to determine if MUC1-C and TWIST1 form a complex on the MUC1 promoter demonstrated that MUC1-C and TWIST1 are both detectable on the region containing the TWIST1 motifs (Fig. 3E, left). Re-ChIP studies further demonstrated that MUC1-C is detectable in a complex with TWIST1 (Fig. 3E, right). Notably, silencing MUC1-C decreased TWIST1 occupancy (Fig. 3F), indicating that MUC1-C directly contributes to activation of TWIST1 on the MUC1 promoter. In concert with these results, we found that silencing TWIST1 is associated with suppression of MUC1-C mRNA levels (Fig. 3G), supporting a model in which MUC1-C and TWIST1 form an autoregulatory loop.

**MUC1-C/TWIST1 circuit promotes EMT and stemness**

EMT-TFs can function cooperatively to induce one another and regulate common sets of target genes (4, 8). In this respect and in addition to TWIST1, silencing MUC1-C was associated...
with suppression of ZEB1 and SNAIL expression (Fig. 4A, left and right). RNA-seq analysis further demonstrated that MUC1-C is highly associated with driving the EMT program as supported by the most significant correlation observed among the different Hallmark gene sets (Fig. 4B; Supplementary Fig. S5 and S6). In addition, Metacore pathway analysis documented significant correlations for MUC1-C (A and C) and TWIST1 (B and D) mRNA levels by qRT-PCR using primers listed in Supplementary Table S1. The results (mean ± SD) are expressed as relative mRNA levels compared with that obtained for control untreated cells (assigned a value of 1). *P < 0.05. Lysates were immunoblotted with antibodies against the indicated proteins (E and F).

Figure 1.
Targeting MUC1-C suppresses TWIST1 expression. A–F, BT-549 and SUM149 cells stably expressing a tet-CshRNA or tet-MUC1shRNA were left untreated or treated with 500 ng/mL DOX for 7 days. Cells were analyzed for MUC1-C (A and C) and TWIST1 (B and D) mRNA levels by qRT-PCR using primers listed in Supplementary Table S1. The results (mean ± SD) are expressed as relative mRNA levels compared with that obtained for control untreated cells (assigned a value of 1). *P < 0.05. Lysates were immunoblotted with antibodies against the indicated proteins (E and F).
We also found that targeting MUC1-C in vivo suppresses expression of SOX2, BMI1, ALDH1, and CD44 (Fig. 4H), supporting a role for MUC1-C in integrating EMT and stemness.

MUC1-C/TWIST1 circuit promotes drug resistance

To investigate the potential role of MUC1-C/TWIST1 signaling in drug resistance, we selected BT-549 cells for survival in the presence of increasing concentrations of paclitaxel (PTX; Supplementary Fig. S13A). As compared with drug naive BT-549 cells, we found partial decreases in MUC1-C levels and upregulation of TWIST1, the ABCB1 drug transporter and SOX2, which also contributes to stemness and drug resistance (ref. 34; Fig. 5A). Immuno-fluorescence microscopy studies showed increased TWIST1 expression in the nucleus (Fig. 5B). In concert with these results, we also detected increases in the occupancy of TWIST1 complexes on the MUC1 (Fig. 5C) and ABCB1 (Fig. 5D) promoters. Importantly, silencing MUC1-C in BT-549/PTX-R cells resulted in downregulation of TWIST1, ABCB1, SOX2, ALDH1, and CD44 (Fig. 5E), and increased sensitivity to PTX treatment (Fig. 5F), indicating that MUC1-C contributes to this program of drug resistance.

In extending these results, BT-549/PTX-R cells were treated with the GO-203 inhibitor, which directly blocks MUC1-C function and is selective against MUC1-C expressing cells (35–37). GO-203 treatment was associated with suppression of MUC1-C, TWIST1, ABCB1, SOX2, BMI1, ALDH1 and CD44 expression (Fig. 6A), further indicating that MUC1-C contributes to drug resistance.

In support of this notion, we found that combining GO-203 and PTX is synergistic against BT-549/PTX-R cells with CI values less than 1.0 (Fig. 6B and C; Supplementary Fig. S13B and S13C; Supplementary Table S3), demonstrating that (i) both agents contribute to loss of viability, and (ii) targeting MUC1-C reverses PTX resistance. Drug resistance is associated with the CSC state (6, 38). In this respect and in concert with the above results, we found that the mammosphere forming capacity of
Figure 3.
MUC1-C forms a complex with TWIST1 on the MUC1 promoter. A, Nuclear lysates were incubated with anti-MUC1-C or a control IgG. The input and precipitates were analyzed by immunoblotting with antibodies against the indicated proteins. B and C, Purified recombinant TWIST1 was incubated with GST or the indicated GST-MUC1-CD fusion proteins. Precipitates obtained with glutathione beads were immunoblotted with anti-TWIST1. Loading of the GST proteins was analyzed by Coomassie Blue staining. D, Schema of the MUC1 promoter with highlighting of the putative TWIST1-binding motifs. E, Soluble chromatin was precipitated with anti-MUC1-C, anti-TWIST1, or a control IgG (left). Soluble chromatin was precipitated with anti-MUC1-C (ChIP) and then re-precipitated with anti-TWIST1 or a control IgG (re-ChIP; right). The DNA samples were amplified by qPCR with primers for the MUC1 promoter. The results (mean ± SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). F, BT-549/tet-MUC1shRNA cells were left untreated or treated with 500 ng/mL DOX for 7 days. Soluble chromatin was precipitated with anti-TWIST1 or a control IgG. The DNA samples were amplified by qPCR with primers for the MUC1 promoter. The results (mean ± SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). G, BT-549/CshRNA and BT-549/TWIST1shRNA cells were analyzed for TWIST1 and MUC1-C mRNA levels by qRT-PCR. The results (mean ± SD) are expressed as relative mRNA levels compared with that obtained for control cells (assigned a value of 1).
BT-549/PTX-R cells is increased as compared with drug-naïve cells (Fig. 6D). Moreover, targeting MUC1-C with silencing (Fig. 6E) or treatment with GO-203 (Fig. 6F) significantly decreased BT-549/PTX-R mammosphere formation. Notably, complete silencing of MUC1-C is not achievable with the tet-MUC1shRNA and, as a result, suppression of mammosphere formation is also not complete (Fig. 6E). In contrast, treatment with GO-203, which suppresses MUC1-C expression in part and inhibits function of remaining MUC1-C by direct binding, completely blocks mammosphere formation (Fig. 6F).

Discussion

EMT has been linked to cancer cell progression, stemness, and drug resistance (2). The cellular plasticity among EMT, MET and their intermediate states contributes to tumor heterogeneity and thereby emergence of more aggressive CSC populations (2). Surprisingly, how these clinically important hallmarks of the cancer cell are interconnected has largely remained unclear (6). The TWIST1 EMT-TF is an essential regulator of cell migration and morphogenesis during embryonic development (27). In contrast...
with ER-positive breast cancer cells that have low to undetectable levels of TWIST1 expression, TWIST1 is upregulated in TNBC cells and promotes their invasion, genetic instability and CSC state (20, 21, 39). The oncogenic MUC1-C protein has been linked to the induction of EMT by activating the NF-κB p65 pathway and in turn expression of the ZEB1 EMT-TF (15). By contrast and importantly, there has been no reported link between MUC1-C and TWIST1. The present studies demonstrate that, unlike ZEB1, MUC1-C induces TWIST1 expression by activation of the proinflammatory STAT3 pathway. MUC1-C has a unique capacity to associate with TFs, such as NF-κB p65 and ZEB1, and to promote their occupancy on target genes (12). Along these lines, we found that MUC1-C induces pSTAT3 in TNBC cells and, in turn, pSTAT3-mediated activation of TWIST1 expression. Mechanistically, MUC1-C and STAT3 form a complex on the TWIST1 promoter and activate TWIST1 transcription. Our results

Figure 5.
MUC1-C drives drug resistance. A, Lysates from drug-naive (WT) BT-549 and BT-549/PTX-R cells were immunoblotted with antibodies against the indicated proteins. B, BT-549 WT and PTX-R cells were analyzed for nuclear MUC1-C and TWIST1 localization by IF microscopy. C and D, Chromatin from BT-549 WT and PTX-R cells was precipitated with anti-MUC1-C, anti-TWIST1, or a control IgG. The DNA samples were amplified by qPCR with primers for the MUC1 (C) and ABCB1 (D) promoters. The results (mean ± SD of three determinations) are expressed as the relative fold enrichment compared with that obtained with the IgG control (assigned a value of 1). E, Lysates from BT-549/PTX-R/tet-MUC1shRNA cells left untreated or treated with 500 ng/mL DOX for 7 days were analyzed by immunoblotting. F, BT-549/PTX-R/tet-MUC1shRNA cells were left untreated or treated with 500 ng/mL DOX for 7 days. The cells were then exposed to vehicle control (CTR) or 30 μmol/L PTX for 72 hours. The results (mean ± SD) are expressed as relative survival as compared with that obtained with DOX-untreated/CTR cells (assigned a value of 1).
further demonstrate that targeting MUC1-C decreases STAT3 occupancy on the TWIST1 promoter and downregulates TWIST1 mRNA and protein. These findings support a previously unrecognized model in which MUC1-C is necessary for pSTAT3-mediated TWIST1 activation (Fig. 6G).

The MUC1-C cytoplasmic domain is an intrinsically disordered protein with the plasticity to directly interact with TFs and epigenetic modulators of gene expression (12). In this way, MUC1-C induces ZEB1 expression and, in turn, binds to ZEB1 with the formation of MUC1-C/ZEB1 complexes on the miR-200c promoter and induction of the EMT program (15). In concert with these findings and the demonstration that MUC1-C drives TWIST1 expression, we also found that MUC1-C directly associates with TWIST1 and that MUC1-C/TWIST1 complexes are constitutively detectable in the nucleus. During the course of these experiments, it became apparent that TWIST1 was in some way reciprocally contributing to the regulation of MUC1-C expression. Indeed, the identification of potential TWIST1 binding motifs in the MUC1 promoter and the demonstration that MUC1-C/TWIST1 complexes occupy those sites, invoked the possibility that MUC1-C and TWIST1 function in an autoregulatory manner. In support of a MUC1-C/TWIST1 circuit, (i) targeting MUC1-C suppressed TWIST1 occupancy on the MUC1 promoter and (ii) silencing TWIST1 downregulated MUC1-C expression (Fig. 6G). These findings support an autoregulatory loop; however, they do not exclude a role for TWIST1 in the fine-tuning of MUC1 activation as may have been observed with upregulation of TWIST1 and partial suppression of MUC1-C in the setting of PTX resistance. Induction of the EMT program is associated with the activation of TWIST1, as well as other EMT-TFs (8, 40).

MUC1-C activates the STAT3 pathway and is necessary for TWIST1 activation. In turn, MUC1-C binds directly to TWIST1 and forms an autoregulatory MUC1-C–TWIST1 circuit. Importantly, our results show that this circuit is sufficient for driving (a) ZEB1 and SNAIL expression, (ii) multiple genes associated with the EMT program and invasion, (iii) BC stem cell markers, (iv) self-renewal and tumorigenicity, and (v) ABCB1 with the acquisition of drug resistance. *P < 0.05.
with a role for MUC1-C in driving expression of multiple EMT-IFs (refs. 15, 41; Fig. 6G). These findings and the results of RNA-seq analyses demonstrating highly significant correlations between MUC1-C and the EMT program provide evidence that MUC1-C contributes to the regulation of multiple genes associated with the EMT state. Our findings that MUC1-C is sufficient to drive EMT and invasive capacity in TNBC cells further support an autocrine process that may be independent of paracrine TGFβ or inflammatory cytokines.

In cancer cells, activation of the EMT program is linked to acquisition of the CSC state, a characteristic consistent with a process of dedifferentiation (42–44). Relatively few insights are available regarding the mechanistic basis for these highly interconnected programs. MUC1-C activates the BMI1 and ALDH1A1 effectors of BC stem cells (17, 45). However, it was not known if MUC1-C contributes to the interconnectivity between EMT and stemness. The present results provide evidence that, in concert with induction of EMT, MUC1-C drives self-renewal capacity. In this way, targeting MUC1-C genetically or pharmacologically suppressed (i) the BC stem cell markers, SOX2, BMI1, ALDH1, and CD44, (ii) mammosphere formation and tumorigenicity, indicating that MUC1-C also promotes stemness. Other evidence has linked MUC1-C to the epigenetic regulation of gene expression by interacting with pathways of effectors, such as DNMTs, BMI1/PRC1 and EZH2/PRC2 (18). Further studies will now be needed to define how MUC1-C may integrate epigenetic modifications with the EMT program, stemness and process of dedifferentiation. EMT and the CSC state are intimately linked to the development of drug resistance (6, 38). In cancer cells, MUC1-C localizes to the mitochondrial outer membrane, where it attenuates the intrinsic apoptotic response to cytotoxic anticancer agents (46–48). MUC1-C also confers resistance to targeted agents, such as tamoxifen and trasutuzumab (49, 50), and contributes to the evasion of immune destruction (51). These findings have collectively supported a potential role for MUC1-C in pleotropic states, such as heterogeneity and stemness, which can protect tumors against treatment. In support of this premise, we found that MUC1-C plays a role in the acquisition of PTX resistance in association with driving expression of TWIST1, ABCB1 and SOX2, BMI1, ALDH1, and CD44. Importantly in this respect, targeting MUC1-C with GO-203 (i) suppressed TWIST1, ABCB1 and the BC stem cell markers, (ii) reversed PTX resistance, and (iii) conferred synergistic activity with PTX against these drug-resistant cells.

Regarding potential clinical relevance, a Phase I trial of GO-203 in patients with solid tumors demonstrated an acceptable safety profile and evidence of antitumor activity. However, a short circulating GO-203 half-life necessitated frequent dosing schedules, which were cumbersome for patients. Accordingly, GO-203 has been reformulated in nanoparticles for weekly administration and further clinical evaluation (52). Antibodies generated against the MUC1-C extracellular domain have also been developed to target MUC1-C—expressing tumors with antibody-drug conjugates and other immune-based therapies (53). When taken in the context of a role for MUC1-C in driving stemness and resistance to treatment, the availability of these agents could provide the basis for designing more effective therapeutic strategies. In summary, our findings represent a previously unrecognized paradigm for MUC1-C—induced integration of TWIST1, EMT, stemness and drug resistance, and support the targeting of MUC1-C for inhibiting cancer progression (Fig. 6G).

Disclosure of Potential Conflicts of Interest

D. Kufe is on the board of directors at Genus Oncology and Hillstream BioPharma; a consultant at Genus Oncology, Reata Pharmaceuticals, CanBas Co. Ltd., and Victa Biotherapeutics; is a scientific advisory board member for Nanogen Therapeutics; and is on the board of directors and has ownership interest (including patents) at Genus Oncology, Reata Pharmaceuticals, Victa Biotherapeutics, Nanogen Therapeutics. No potential conflicts of interest were disclosed by the other authors.

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