Human Mucin 1 Oncoprotein Represses Transcription of the *p53* Tumor Suppressor Gene

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Abstract

The mucin 1 (MUC1) heterodimeric protein is aberrantly overexpressed in human breast cancers and induces transformation. The MUC1 COOH-terminal subunit (MUC1-C) is targeted to the nucleus of transformed cells, where it interacts with *p53* and regulates p53-mediated transcription. The present studies show that MUC1 represses activation of the *p53* gene and that MUC1-C occupies the PE21 element in the *p53* proximal promoter. Previous work has shown that the Kruppel-like factor 4 (KLF4) transcription factor represses *p53* transcription by binding to the PE21 element. Our results show that MUC1-C binds constitutively to KLF4, occupies PE21 with KLF4, and enhances the KLF4 occupancy of PE21. The results also show that MUC1-C increases the recruitment of histone deacetylases 1/3, deacetylation of core histones, and repression of *p53* transcription. These findings indicate that overexpression of MUC1, as found in human breast cancer cells, is of functional importance to repression of the *p53* gene. [Cancer Res 2007;67(4):1853–8]

Introduction

The human mucin 1 (MUC1)–type glycoprotein is expressed on the apical borders of normal secretory mammary epithelial cells (1). MUC1 is translated as a single polypeptide and undergoes autoproteolysis into two subunits that form a heterodimeric complex (2–4). The MUC1 NH_2-terminal subunit (MUC1-N) contains variable numbers of 20 amino acid tandem repeats that are heavily modified with *O*-linked glycans (5, 6). The MUC1 COOH-terminal subunit (MUC1-C) includes a 58–amino acid extracellular domain, a 28–amino acid transmembrane domain, and a 72–amino acid cytoplasmic tail (7). With transformation and loss of polarity, MUC1 is expressed at high levels over the entire surface of breast carcinoma cells, a setting in which MUC1 interacts with the ErbB and FGFR3 receptor tyrosine kinases (1, 8–12). In addition, MUC1-C accumulates in the cytosol and is targeted to the nucleus and mitochondria (13–15). Importantly, overexpression of MUC1-C, and not the MUC1-N mucin component, is sufficient to induce transformation and resistance to stress-induced apoptosis (11, 13, 16–18). MUC1-C binds directly to and stabilizes β-catenin, and thereby contributes to the activation of Wnt target genes (11, 19–21). Nuclear MUC1-C also interacts with p53 and regulates p53-dependent activation of the *p21* gene in the response to genotoxic stress (22). These findings have supported a role for MUC1-C in promoting growth and survival of the 80% to 90% of human breast cancers that overexpress this oncoprotein.

The Kruppel-like factor (KLF) family of transcription factors is characterized by the presence of three Kruppel-like zinc fingers and includes the SP1-like proteins (23). Like certain other family members, KLF4 (GKLF/EZF) acts as both an activator and repressor of genes involved in cell cycle regulation (24). As such, KLF4 functions as a tumor suppressor by inhibiting the proliferation of nontransformed cells. Paradoxically, KLF4 also functions as a suppressor of p53 expression by acting directly on the PE21 element in the *p53* promoter (25). In this context, KLF4 promotes transformation and resistance to DNA damage–induced apoptosis (25). The available evidence indicates that the oncogenic function of KLF4 emerges in the presence of cyclin D1 signaling or in the absence of p21 (24). KLF4 is also required for p53-mediated induction of p21 in the growth arrest response to DNA damage (26, 27). Moreover, the demonstration that KLF4 associates with p53 has indicated that KLF4 could directly affect the p53 transactivation function (26, 28). Notably, KLF4 is overexpressed in up to 70% of human breast cancers (29) and nuclear localization of KLF4 is associated with an aggressive phenotype (30). In addition, silencing of KLF4 in human breast cancer cells is associated with the elevation of endogenous p53 levels and the induction of apoptosis (25), findings consistent with a KLF4 oncogenic function.

The overexpression of MUC1 and KLF4 in human breast cancers and the importance of both proteins in the regulation of p53 prompted us to investigate whether MUC1 interacts with KLF4. The results show that MUC1-C constitutively associates with KLF4 and that this interaction is of functional significance to repression of the *p53* gene.

Materials and Methods

Cell culture. MCF-7 breast cancer cells and those stably infected with a control small interfering RNA (MCF-7/CsiRNA) or one expressing a MUC1siRNA (MCF-7/MUC1siRNA) were grown in DMEM with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 μg/mL of streptomycin, 100 units/mL of penicillin, and 2 mmol/L of L-glutamine. Human ZR-75-1 breast cancer cells and those stably infected with a control siRNA (ZR-75-1/CsiRNA) or one expressing a MUC1siRNA (ZR-75-1/MUC1siRNA; ref. 31) were cultured in RPMI 1640 supplemented with 10% HI-FBS, 100 μg/mL of streptomycin, 100 units/mL of penicillin, and 2 of mmol/L L-glutamine. Cells were treated with 50 μmol/L of etoposide (Sigma, St. Louis, MO).

Immunoblotting. Lysates were prepared from subconfluent cells as described (31). Immunoblot analysis was done with anti-p53 (Ab-2 and Ab-6; Oncogene Research Products, San Diego, CA), anti–MUC1-C (Ab-5; Neomarkers, Fremont, CA), anti-KLF4 (H-180; Santa Cruz Biotechnology, Santa Cruz, CA), anti–β-actin (Sigma), anti-IκBα (Santa Cruz Biotechnology), or anti–proliferating cell nuclear antigen (F-2; Santa Cruz Biotechnology). Lysates were also first subjected to immunoprecipitation with anti-KLF4 and the precipitates were analyzed by immunoblotting. Immunocomplexes were detected with enhanced chemiluminescence (Perkin-Elmer Life Sciences, Boston, MA).

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

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Transfection and reporter assays. Transfections were done in 60 mm dishes using Fugene-6 (Roche Applied Science, Indianapolis, IN) or, for the luciferase assays, in 24-well plates using the calcium phosphate method (Invitrogen, San Diego, CA). Cells were transfected with the −2400-p53-Luc reporter, −2400-PE21-MUT-Luc reporter, −320-p53-Luc reporter, −320-PE21-MUT-Luc reporter (25), and an internal control LacZ expression plasmid (pCMV-LacZ; ref. 32). Luciferase assays were done with the Luciferase Assay System (Promega, Corp., Madison, WI) at 40 h after transfection. Luciferase activity was normalized to that obtained for LacZ and presented as relative luciferase activity.

Glutathione *S*-transferase pull-down assays. Glutathione *S*-transferase (GST) and GST fusion proteins were purified by binding to glutathione-agarose beads (Sigma). ^{35}S-labeled KLF4 prepared in TNT reactions (Promega) was incubated with GST or the GST fusion proteins for 2 h at 4°C. After washing, the adsorbed proteins were resolved by SDS-PAGE and analyzed by autoradiography.

Chromatin immunoprecipitation and Re-ChIP assays. Chromatin immunoprecipitation (ChIP) assays were done as described (33) using anti-MUC1-C, anti-KLF4, anti-HDAC1 (Upstate Biotechnology, Inc., Lake Placid, NY), anti-HDAC3 (Upstate Biotechnology), anti–Ac-H3 (Upstate Biotechnology), or anti–Ac-H4 (Upstate Biotechnology). For Re-ChIP assays, complexes from the primary ChIP were eluted with 10 mmol/L of DTT for 30 min at 37°C, diluted 20 times with Re-ChIP buffer [20 mmol/L Tris-HCl (pH 8.1), 1% Triton X-100, 2 mmol/L EDTA, 150 mmol/L NaCl] followed by reimmunoprecipitation with the indicated second antibodies and were again subjected to the ChIP procedure. The final DNA extractions were amplified by PCR using primers that covered the *p53* proximal promoter (PP; −118 to +14), the PE21 element (PE21; −118 to −54), and a control region (CR; −6020 to −5940). For PCR, 2 μL from a 50 μL DNA extraction were used with 30 to 38 cycles of amplification. The primers for the *p53* proximal promoter (PP) were (5′-GCCCTTACTTGTCATGGCGA; 3′-GGCTCTAGACTTTTGAGAAGC). The primers for the PE21 region that covers PE21 motif were (5′-GCCCTTACTTGTCATGGCGA; 3′-CAATCCCATCAACCCCTGC) as described (25). The primers for the *p53* control region (CR) will be (5′-TGACCTCAGGCGATCCACCTG; 3′-GCACTTAAGGCCGGGTGCGGT).

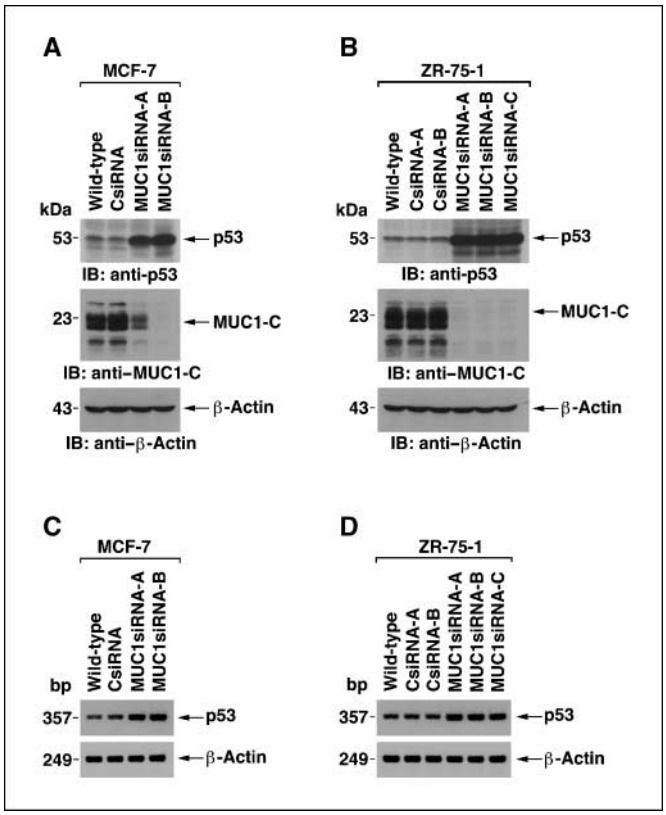

Figure 1. MUC1 down-regulates p53 at protein and mRNA levels. *A* and *B,* lysates from the indicated MCF-7 (*A*) and ZR-75-1 (*B*) cells were immunoblotted with anti-p53, anti–MUC1-C, and anti–β-actin. *C* and *D,* semiquantitative reverse transcriptase-PCR for p53 and anti–β-actin mRNA levels were done on the indicated MCF-7 (*C*) and ZR-75-1 (*D*) cells.

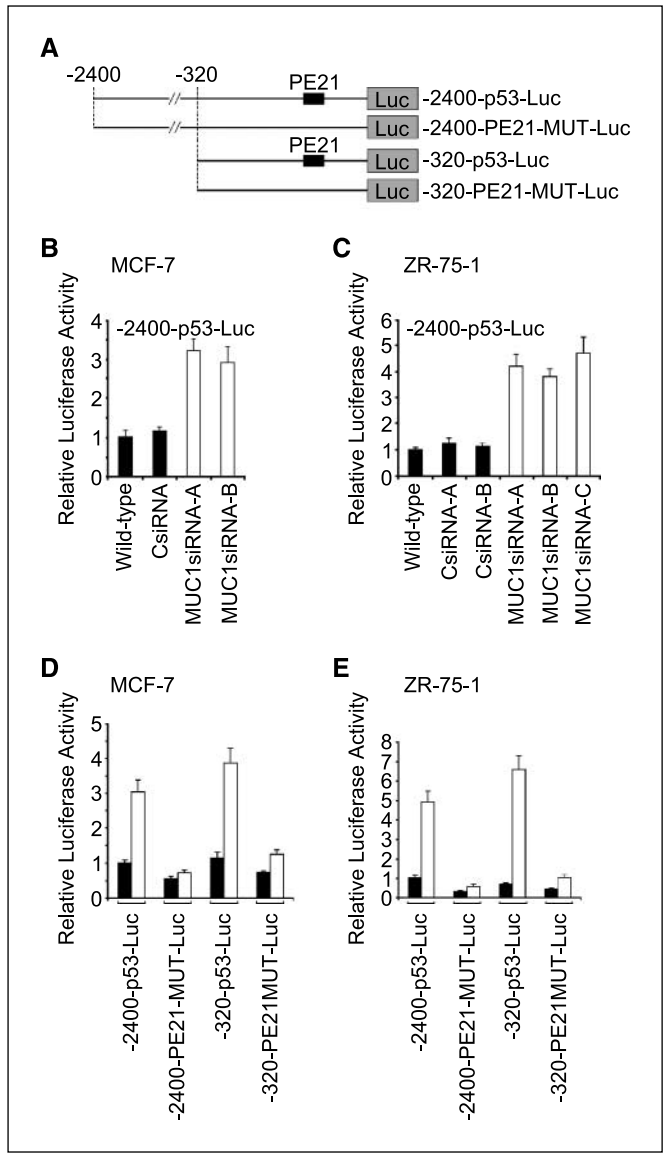

Figure 2. MUC1 suppresses *p53* gene transcription. *A,* schematic depiction of the human *p53* gene promoter and the mutants used in this study. *B* to *E,* MCF-7/CsiRNA cells (*filled columns*) and MCF-7/MUC1siRNA (*open columns*; *B* and *D*), or ZR-75-1/CsiRNA cells (*filled columns*) and ZR-75-1/MUC1siRNA (*open columns*; *C* and *E*) were transfected with the −2400-p53-Luc reporter, −2400-PE21-MUT-Luc reporter, −320-p53-Luc reporter, −320-PE21-MUT-Luc reporter, and an internal control LacZ expression plasmid (pCMV-LacZ). At 40 h after transfection, the cells were assayed for luciferase activity. The results are expressed as the fold activation (mean ± SD of three separate experiments) compared with that obtained with wild-type cells (assigned a value of 1).

Results and Discussion

MUC1 down-regulates p53 mRNA and protein levels. To determine whether MUC1 regulates p53 expression, human MCF-7 breast cancer cells that express endogenous MUC1 were stably

infected with a retrovirus expressing a MUC1siRNA. Immunoblot analysis of two separately isolated MCF-7/MUC1siRNA clones showed that silencing MUC1 was associated with increases in p53 as compared with that in wild-type cells and cells expressing a CsiRNA (Fig. 1*A*). Similarly, silencing MUC1 in human ZR-75-1 breast cancer cells was associated with increases in p53 expression (Fig. 1*B*). In addition and to exclude off-target effects, the cells were transfected with a pool of MUC1siRNAs (Dharmacon SMARTpool Reagents, Lafayette, CO). The results show that transiently silencing MUC1 increases p53 expression (Supplemental Fig. S1; data not shown). Immunoblot analysis of purified nuclear and cytosolic fractions from the MCF-7 and ZR-75-1 cells showed that silencing MUC1 was associated with increases in p53 expression in the cytoplasm and nucleus (Supplemental Figs. S2*A* and *B*). Semiquantitative reverse transcriptase-PCR analysis of the MCF-7 and ZR-75-1 cells showed that p53 mRNA levels were increased by silencing MUC1 (Fig. 1*C* and *D*). The p53 protein was stabilized in response to DNA damage (34). Treatment of MUC1-positive ZR-75-1/CsiRNA cells with the genotoxic agent, etoposide, showed that p53 levels increase in response to DNA damage (Supplemental Fig. S3*A*). However, the p53 levels in ZR-75-1/CsiRNA cells remained substantially lower than that found in the ZR-75-1/MUC1siRNA cells (Supplemental Fig. S3*A*). Similar results were obtained in the MCF-7/CsiRNA and MCF-7/MUC1siRNA cells (data not shown). These findings and analysis of p53 mRNA levels indicate that MUC1 down-regulates p53, at least in part, by a transcriptional mechanism.

MUC1 suppresses *p53* gene transcription. To determine if MUC1 regulates activation of the *p53* promoter, cells without or with MUC1 silencing were transfected with a *p53* promoter-Luc reporter (−2400-p53-Luc; Fig. 2*A*) and an internal control LacZ expression plasmid (pCMV-LacZ). Results of luciferase assays showed that *p53* promoter activity was decreased in MCF-7/CsiRNA expressing endogenous MUC1 as compared with that in MUC1-silenced MCF-7/MUC1siRNA cells (Fig. 2*B*). Similar results were obtained with the ZR-75-1/CsiRNA and ZR-75-1/MUC1siRNA

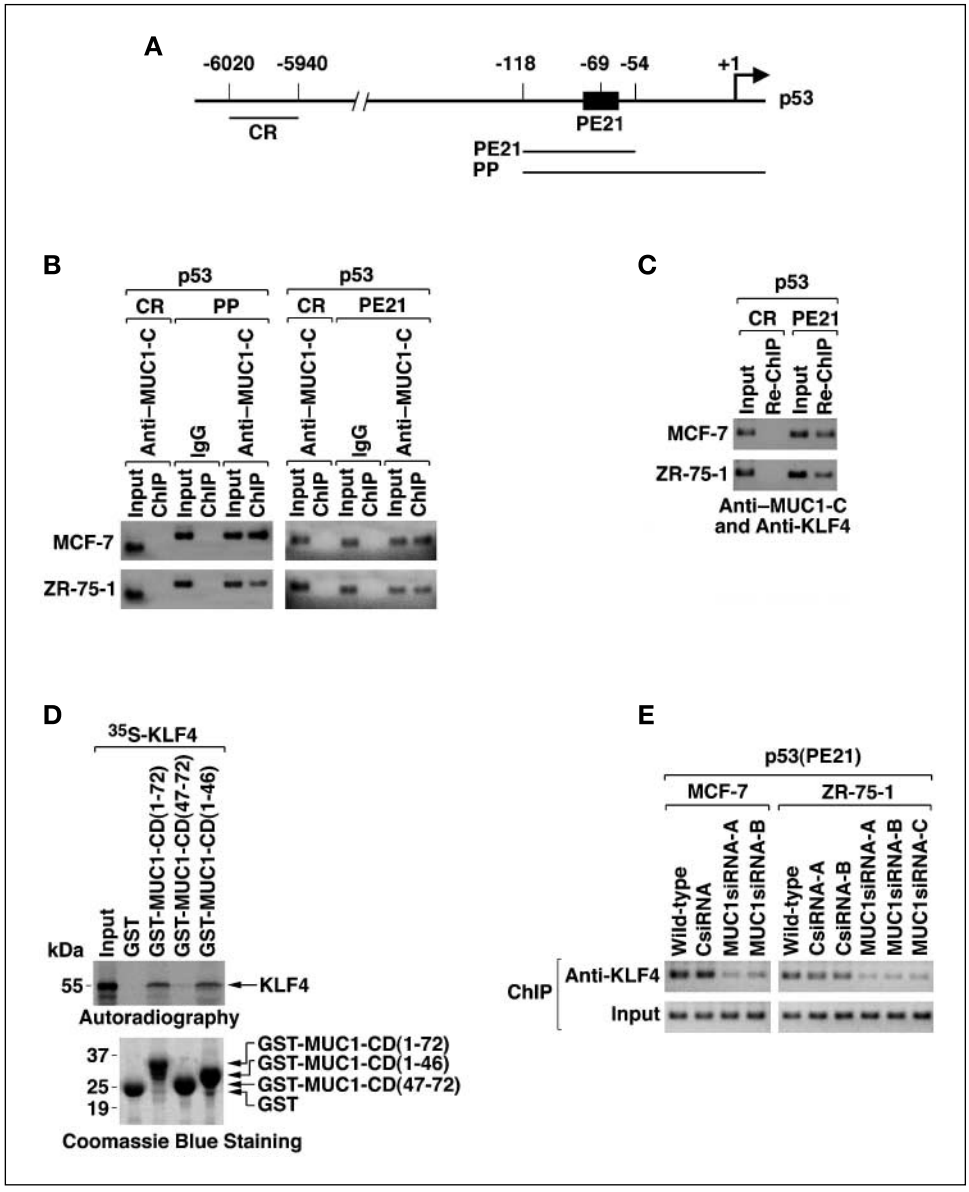

Figure 3. MUC1 occupies the *p53* gene proximal promoter with KLF4. *A,* schema depicting the structure of *p53* gene promoter. *B,* soluble chromatin from MCF-7 and ZR-75-1 cells was immunoprecipitated with anti–MUC1-C or a control IgG. The final DNA extractions were amplified by PCR using primers that cover a control region (*CR*; −6020 to −5940), the proximal promoter (*PP*; −118 to +14), or the region that contains the PE21 element (*PE21*; −118 to −54) in the *p53* gene promoter. *C,* in Re-ChIP experiments, soluble chromatin from MCF-7 or ZR-75-1 cells was immunoprecipitated with anti–MUC1-C, eluted with DTT, diluted with Re-ChIP buffer, reimmunoprecipitated with anti-KLF4, and analyzed for *p53* PE21 sequences. *D,* GST, GST-MUC1-CD (1–72), or the indicated GST-MUC1-CD deletion mutants were bound to glutathione agarose and incubated with [^{35}S]-labeled KLF4. The adsorbates were analyzed by SDS-PAGE and autoradiography. Input of the GST and GST-MUC1-CD fusion proteins was assessed by Coomassie blue staining. *E,* soluble chromatin from MCF-7 and ZR-75-1 cells was immunoprecipitated with anti–MUC1-C or a control IgG. The final DNA extractions were amplified by PCR using primers that cover a control region (CR; −6020 to −5940) or the region that contains the PE21 element (PE21; −118 to −54) in the *p53* gene promoter.

cells (Fig. 2*C*), indicating that MUC1 represses the activity of the *p53* promoter. The PE21 element in the proximal promoter of the *p53* gene has been shown to confer suppression of *p53* transcription (25, 35). To determine whether the PE21 element was required for MUC1-mediated suppression, MCF-7 and ZR-75-1 cells without or with MUC1 silencing were transfected with *p53* promoter-Luc (−2400-p53-Luc) or the reporter with a mutant PE21 element (−2400-PE21-MUT-Luc; Fig. 2*A*). The increase in *p53* promoter activity in MCF-7 cells silenced for MUC1 was abrogated by mutating the PE21 element (Fig. 2*D*). Similar results were obtained when using the −320-p53-Luc or −320-PE21-MUT-Luc (Fig. 2*D*). Activation of the *p53* promoter in ZR-75-1 cells silenced for MUC1 was also abrogated by mutating the PE21 element in both −2400-p53-Luc and −320-p53-Luc (Fig. 2*E*), indicating that the PE21 element was required for MUC1-mediated suppression of the *p53* promoter.

MUC1 occupies the *p53* proximal promoter. To study if MUC1 binds to the *p53* promoter, ChIP assays were done on the *p53* proximal promoter (PP; −118 to +14) with an anti–MUC1-C antibody (Fig. 3*A*). MUC1 occupancy of the *p53* proximal promoter was detectable in anti–MUC1-C and not in control IgG precipitates (Fig. 3*B, left*). In addition, there was no detectable MUC1 associated with a control region (CR; −6020 to −5940) upstream of the *p53* proximal promoter (Fig. 3*A*). To determine whether MUC1 binds to the PE21 element (−79 to −59), ChIP analyses were done using primers that cover the *p53* promoter region from −118 to −54 (designated PE21 region; Fig. 3*A*). MUC1 occupancy of the PE21 region was detectable in anti–MUC1-C, and not the control IgG, precipitates from MCF-7 and ZR-75-1 cells (Fig. 3*B, right*). These results indicate that MUC1 occupies the PE21 region and thereby contributes to the suppression of *p53* gene transcription. KLF4 suppresses *p53* gene transcription by occupying the PE21 element of the *p53* gene promoter (25). To determine if MUC1-C occupies the PE21 region with KLF4, Re-ChIP assays were done using anti–MUC1-C and anti-KLF4 antibodies. Analysis of MCF-7 and ZR-75-1 cells showed that anti-KLF4 precipitates the PE21 region after their

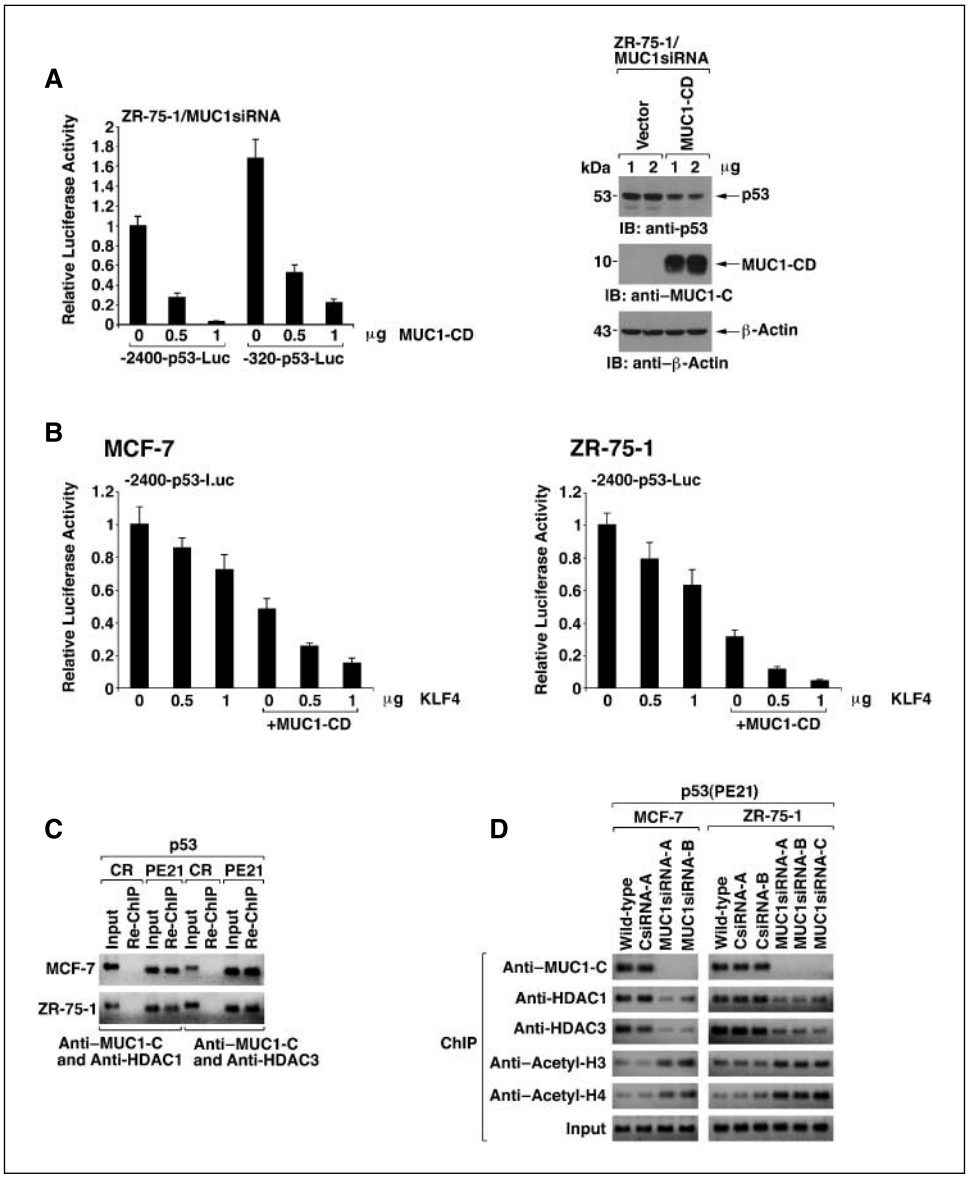

Figure 4. MUC1 suppresses *p53* gene transcription by recruiting HDACs to its promoter. *A,* ZR-75-1/MUC1siRNA cells were transfected with the −2400-p53-Luc reporter, −320-p53-Luc reporter, the indicated amounts of MUC1-CD and an internal control LacZ expression plasmid (pCMV-LacZ). At 40 h after transfection, the cells were assayed for luciferase activity. The results are expressed as the fold activation (mean ± SD of three separate experiments) compared with that obtained with empty vector–transfected cells (assigned a value of 1; *left*). ZR-75-1/MUC1siRNA cells were transfected with the indicated mounts of pCMV or pCMV-MUC1-CD vectors. At 24 h after transfection, lysates from the indicated ZR-75-1/MUC1siRNA cells were immunoblotted with anti-p53, anti–MUC1-C, and anti–β-actin (*right*). *B,* MCF-7 (*left*) or ZR-75-1 (*right*) cells were transfected with the −2400-p53-Luc reporter, the indicated amounts of KLF4, 0.5 µg MUC1-CD and an internal control LacZ expression plasmid (pCMV-LacZ). At 40 h after transfection, the cells were assayed for luciferase activity. The results were expressed as the fold activation (mean ± SD of three separate experiments) compared with that obtained with empty vector–transfected cells (assigned a value of 1). *C,* in Re-ChIP experiments, soluble chromatin from MCF-7 or ZR-75-1 cells was immunoprecipitated with anti–MUC1-C, eluted with DTT, diluted with Re-ChIP buffer, reimmunoprecipitated with anti-HDAC1 or anti-HDAC3, and analyzed for *p53* PE21 sequences. *D,* in ChIP experiments, soluble chromatin from MCF-7 and ZR-75-1 cells was immunoprecipitated with the indicated antibodies. The final DNA extractions were analyzed for *p53* PE21 sequences by the amplification of PCR.

release from anti–MUC1-C, indicating that MUC1-C occupies the PE21 region with KLF4 (Fig. 3*C*). In concert with these results, we found that MUC1-C coprecipitates with KLF4 (Supplemental Fig. S3*B*). Moreover, DNA damage had little if any effect on this interaction (Supplemental Fig. S3*B*). To determine if MUC1-C binds directly to KLF4, GST, GST-MUC1-CD, or GST-MUC1-CD deletion fusion proteins were incubated with [^{35}S]-labeled KLF4. Analysis of adsorbates to glutathione beads showed that KLF4 binds to MUC1-CD(1–72) and MUC1-CD(1–46), but not with MUC1-CD(47–72; Fig. 3*D*). These results indicate that KLF4 forms complexes with MUC1-C in cells by binding directly to the MUC1-CD NH_2-terminal region (amino acids 1–46). ChIP assays were also done with anti-KLF4 to assess whether MUC1 affects KLF4 occupancy of the *p53* promoter. Notably, silencing MUC1 was associated with decreased occupancy of the PE21 region by KLF4 (Fig. 3*E*). By contrast, MUC1 silencing had no apparent effect on total cell KLF4 levels (Supplemental Fig. S4), indicating that MUC1-C increases KLF4 occupancy of the PE21 region.

MUC1-CD potentiates KLF4-mediated repression of *p53* transcription. To determine if MUC1 affects the activation of the *p53* promoter, ZR-75-1/MUC1siRNA cells were transfected with −2400-p53-Luc or −320-p53-Luc and MUC1-CD. Of note, the MUC1siRNA used to silence MUC1 in the ZR-75-1 cells targets the extracellular region of MUC1-C and not the cytoplasmic domain (14). Results of the luciferase assays showed that MUC1-CD suppresses *p53* gene transcription (Fig. 4*A, left*). Immunoblot analysis further showed that MUC1-CD down-regulates p53 levels (Fig. 4*A, right*). MCF-7 and ZR-75-1 cells were also transfected with −2400-p53-Luc, MUC1-CD, and increasing amounts of KLF4. The results confirmed that MUC1-CD potentiates KLF4-mediated suppression of *p53* transcription (Fig. 4*B*). Histone deacetylases (HDAC) are a family of enzymes involved in transcriptional repression by catalyzing the deacetylation of core histones (36, 37). To determine if MUC1 occupies the PE21 region with HDACs, Re-ChIP assays were done using anti–MUC1-C, anti-HDAC1, and HDAC3 antibodies. Analysis of MCF-7 and ZR-75-1 cells showed that anti-HDAC1 precipitates the PE21 region after release from anti–MUC1-C, indicating that MUC1-C occupies the region with HDAC1 (Fig. 4*C*). The results also show that MUC1 occupies the PE21 region with HDAC3 (Fig. 4*C*). Recruitment of HDACs plays an essential role in transcriptional repression by catalyzing the deacetylation of acetylated core histones (36, 37). ChIP assays from MCF-7 cells showed that the occupancy of the PE21 region by HDAC1 and HDAC3 is higher in MCF-7/CsiRNA cells, which express endogenous MUC1, as compared with MUC1-negative, MCF-7/MUC1siRNA cells (Fig. 4*D, left*). We also found that MUC1 decreases the acetylation of histone 3 and histone 4 in MCF-7/CsiRNA, as compared with MCF-7/MUC1siRNA cells (Fig. 4*D, left*). Similar results were obtained in the ZR-75-1 cells (Fig. 4*D, right*). These findings indicate that MUC1 represses the activity of the *p53* promoter by the recruitment of HDACs to the PE21 element and thereby deacetylation of histones.

MUC1 regulates both p53 function and expression. Previous work showed that MUC1-C binds directly to p53 and coactivates p53-mediated transcription of the *p21* gene (22). MUC1-C also occupies the *Bax* proximal promoter that includes the TATA box and, in contrast to *p21*, represses *Bax* gene transcription by disrupting the assembly of the basal transcription apparatus (22). The human *p53* promoter does not have a TATA or GC box (35, 38). However, the PE21 element within the *p53* proximal promoter directs bidirectional initiation activity as found with TATA and GC boxes (35, 39, 40). The PE21 element functions as a binding site for KLF4, a repressor of p53 transcription that transforms cells as a function of p21 status (24, 25, 35). The present results show that MUC1-C binds to KLF4, occupies the PE21 region constitutively with KLF4, increases KLF4 occupancy of PE21, and suppresses *p53* gene transcription in the absence of DNA damage (Fig. 5). We also found that MUC1 contributes to the recruitment of HDAC1/3, deacetylation of core histones and repression of *p53* transcription (Fig. 5). These results indicate that, in addition to regulating the p53 transcription function, MUC1-C acts by suppressing p53 expression. Importantly, like MUC1 (1), KFL4 is overexpressed in the majority of human breast tumors (29, 30, 41). Previous studies have shown that silencing MUC1 in MCF-7 and ZR-75-1 breast cancer cells is associated with decreases in cell growth and increases in cell death (31). Other work has shown that silencing MUC1 is associated with increases in DNA damage–induced apoptosis (22). Silencing KLF4 in breast cancer cells is also associated with the induction of apoptosis (25). Thus, the interaction between MUC1-C and KFL4 in repressing the activation of the *p53* gene may be of importance in the development of human breast cancer.

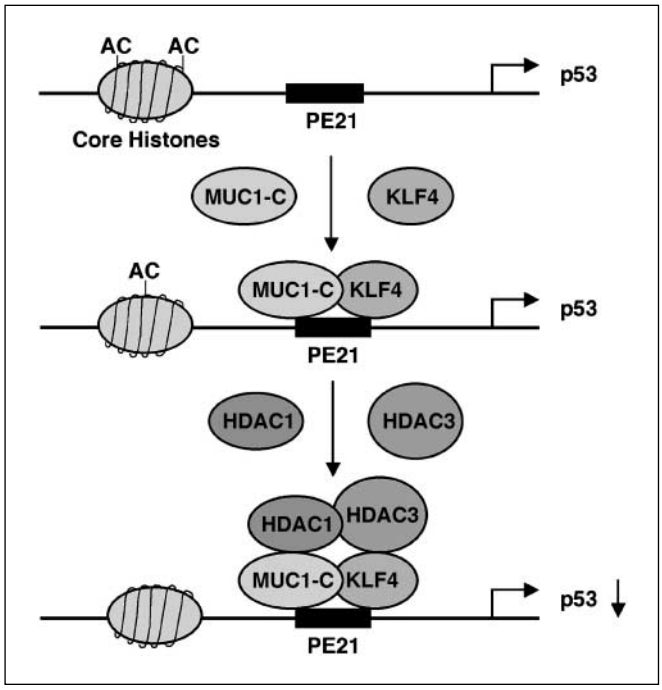

Figure 5. Schema depicting the down-regulation of *p53* gene transcription by MUC1.

Acknowledgments

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