

Research Paper

# Suppression of MUC1 Synthesis Downregulates Expression of the Epidermal Growth Factor Receptor

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## KEY WORDS

Mucin, MUC1, Epidermal growth factor, RNA interference

## ABBREVIATIONS

EGFR	epidermal growth factor receptor
CT	cytoplasmic tail
RNAi	RNA interference
siRNA	small interfering RNA
DMEM	Dulbecco's Modified Eagle's medium

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## ABSTRACT

The transmembrane mucin, MUC1, is overexpressed on many human carcinoma cells, increasing their metastatic potential through decreased cell-cell and cell-matrix adhesion. These cellular changes are mediated both through the altered physical properties of the mucin itself and through the role of the MUC1 cytoplasmic domain as a signaling molecule. The epidermal growth factor receptor (EGFR) is also overexpressed in many cancers and both it and MUC1 constitute important therapeutic targets. In the present study, expression of MUC1 was downregulated by treatment of KB carcinoma cells with a MUC1 small interfering RNA resulting in an inhibition of cell proliferation and colony formation and an increase in cell-cell aggregation. Surprisingly, suppression of MUC1 also inhibited expression of EGFR at both the mRNA and protein levels whereas the reciprocal effect was not observed. These results demonstrate a role for MUC1 in the regulation of EGFR expression and suggest that MUC1 gene silencing may represent a novel therapeutic approach in the treatment of a variety of human cancers.

## INTRODUCTION

MUC1 is a membrane-associated glycoprotein consisting of two noncovalently associated subunits, an N-terminal mucin-like subunit containing a variable number of highly O-glycosylated 20 amino acid tandem repeats and a C-terminal subunit containing a short extracellular domain, a transmembrane domain and a 72-amino acid cytoplasmic tail (CT).<sup>1,2</sup> MUC1 is normally expressed on the apical surfaces of epithelial cells, but is significantly overexpressed in many tumors and malignant cells with underglycosylated forms occurring over the entire cell surface (reviewed in ref. 3). This expression pattern has been shown to contribute to inhibition of both cell-cell adhesion and interaction of cells with the extracellular matrix.

While the highly glycosylated extracellular domain of MUC1 mediates these anti-adhesive properties, increasing evidence suggests that the cytoplasmic subunit of MUC1 also plays an important role in this and other processes. The MUC1 CT contains binding sites for Grb2/Sos and  $\beta$ -catenin and can be phosphorylated by a variety of kinases including the epidermal growth factor receptor (EGFR), c-Src and GSK-3 $\beta$ .<sup>4-7</sup> Phosphorylation by EGFR or c-Src increases the binding of MUC1 CT to  $\beta$ -catenin leading to a reduction in the binding of  $\beta$ -catenin to E-cadherin and an inhibition of E-cadherin-mediated cell adhesion.<sup>6,7</sup> The MUC1 CT- $\beta$ -catenin complex has been localized both in the cytoplasm and in the nucleus<sup>8,9</sup> and it has been suggested that the cytoplasmic domain of MUC1 may be involved in transcriptional regulation as well as intracellular signaling.<sup>10</sup> In addition, transfection of fibroblasts with MUC1 CT lead to anchorage independent growth and tumor formation in nude mice suggesting that it can function as an oncogene.<sup>9,10</sup>

The erbB family of transmembrane receptor tyrosine kinases (erbB1 [or EGFR], erbB2, erbB3 and erbB4) is also involved in the development and progression of a wide range of human cancers.<sup>11</sup> Homo- and heterodimerization of these molecules in response to ligand binding leads to the activation of a number of different adaptor proteins and signaling cascades including those mediated by the MAP kinases ERK1/2, p38 and JNKs.<sup>12</sup> Like MUC1, EGFR is overexpressed in many human cancers and can function as an oncogene by conferring growth in soft agar and promoting tumor formation in nude mice.<sup>13,14</sup> It is of interest in this regard that MUC1 has been shown to participate in a physical association with EGFR as well as to serve as a substrate for EGFR phosphorylation.<sup>6,7</sup>

Thus, both MUC1 and EGFR have become attractive targets for several types of novel therapeutic strategies in different types of cancer. MUC1 and EGFR-specific antibodies have been used in an increasing number of preclinical studies and clinical trials, with

promising results (reviewed in refs. 15–17). Alternative strategies using antisense oligonucleotides or constructs specific for MUC1<sup>18</sup> and EGFR<sup>19,20</sup> have also been used. However, a more promising approach for downregulating these molecules may be the selective degradation of the target mRNAs by RNA interference (RNAi).

In the present study, the effects of MUC1 and EGFR siRNAs on the behavior of KB oral epidermoid carcinoma cells were examined. Suppression of MUC1 was found to inhibit colony formation to an extent even greater than that achieved with EGFR suppression. Surprisingly, cells in which MUC1 was suppressed demonstrated a concomitant decrease in EGFR expression, whereas the reciprocal effect was not observed. These results demonstrate a previously unrecognized role for MUC1 in regulating EGFR expression and suggest that siRNA-mediated inhibition of MUC1 with induced downregulation of the EGFR tyrosine kinase may constitute a novel therapeutic approach in the treatment of human cancer.

## MATERIALS AND METHODS

**Cell culture.** KB (Human oral epidermoid carcinoma) cells were obtained from ATCC (Rockville, MD) and maintained in Dulbecco's Modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Invitrogen), penicillin (100 units/ml) and streptomycin (50 mg/ml) in 5% CO<sub>2</sub> at 37°C. AGS (human gastric adenocarcinoma) and 293 (human embryonic kidney) cells were kindly provided by Dr. Zhiyi Chen, Boston University Medical Center and were grown in the same medium.

**Small interfering RNA (siRNA) treatment.** KB cells were seeded in DMEM containing serum in a 24-well plate and allowed to adhere overnight. The medium was replaced with Opti-MEM reduced serum medium (Invitrogen), and cells were transfected with siRNA duplexes (0.8 µg) using TransMessenger reagent (Qiagen, Valencia CA). Duplexes used were MUC1 siRNA 1 (sense, GAUCCAGCACCGACUACUdTdT; antisense, AGUAGUCGGUGCUGGGAUcDdTdT); MUC1 siRNA 2 (sense, GUUCAGUGCCCAGCUCUACdTdT; antisense, GUAGAGCUGGGCA-CUGAACdTdT), control (nonsilencing) siRNA duplex (sense, UUCUC-CGAACGUGUCACGUdTdT; antisense, ACGUGACACGUUCGGAGA-AdTdT) (Qiagen). In other experiments, cells were treated with Lamin A/C siRNA (Qiagen) and EGFR and erbB2 Smart Pool siRNAs (Upstate Biotechnology, Lake Placid, NY). Four hours after transfection, cells were washed with PBS, DMEM containing serum and antibiotics was added and cells were incubated for an additional 48 h.

**Real time PCR.** RNA was isolated from control and treated cells using Tripure reagent (Roche, Indianapolis, IN) and cDNA was synthesized using standard procedures. Real time PCR was carried out in the presence of SYBR green as described previously<sup>21</sup> to measure levels of MUC1 and EGFR mRNAs. The forward and reverse primers used to generate a 165 bp MUC1 amplicon were: 5'CCAGCCGGGATACCTACCATCCTATGAG and 5'AGAAGTGGCTGCCACTGCTGGGTTTG, respectively. The forward and reverse primers used to generate a 283 bp EGFR amplicon were: 5'GTCAAGTGCTGGATGATAGACGCA and 5'CTGGTTGCACTCAGAGACTCAGG, respectively. Levels (number of molecules) of MUC1

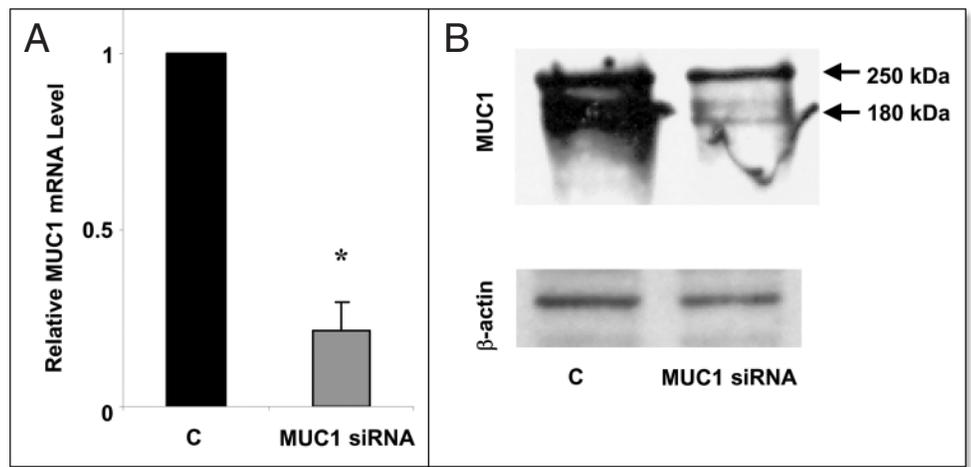


Figure 1. MUC1 siRNA reduces expression of MUC1 transcripts and protein. (A) KB cells were transfected with nonsilencing control siRNA (C) or MUC1 siRNA, RNA was isolated 48 h after transfection and MUC1 transcript levels were measured by real-time PCR and normalized to levels of  $\beta$ -actin transcripts. (B) Cell lysates from identically treated cells were electrophoresed and blots were probed with an antibody directed against the extracellular subunit of MUC1. Blots were stripped and reprobed with an antibody against  $\beta$ -actin. Protein bands corresponding to the extracellular subunit of MUC1 are indicated with arrows.

and EGFR mRNAs were determined from standard curves and normalized to levels of  $\beta$ -actin (231 bp amplicon) determined using forward and reverse primers 5'GCGGGAAATCGTGCGTGACATT and 5'GATGGAGTTGAGGTTAGTTTCGTG.

Real-time PCR was performed on a DNA Engine Opticon System (MJ Research, Waltham, MA) using the cycle profile: 1 cycle at 95°C for 15 min followed by 40 cycles of 95°C for 30 sec; 60°C (for MUC1), 53°C (for EGFR) or 50°C (for  $\beta$ -actin) for 30 sec; and 72°C for 30 sec (for MUC1 and EGFR) or 20 sec (for  $\beta$ -actin). Standard curves for MUC1, EGFR and  $\beta$ -actin were generated using serially diluted templates of known copy number. Melting curve analysis was performed to verify the specificity of the amplification reactions and reactions were performed in triplicate.

**Cell lysate preparation and Western blots.** Cells were lysed on ice in RIPA buffer and soluble protein fractions were prepared as described previously.<sup>21</sup> The concentration of protein in the lysates was determined using the BCA protein assay (Pierce, Rockford, IL) and aliquots (25–50 µg) were electrophoresed on 8% polyacrylamide gels. Proteins were transferred to nitrocellulose membranes and blots were blocked and incubated with primary antibodies for 1 h at room temperature. A monoclonal MUC1 antibody (MUC1/EMA; US Biological, Swampscott, MA) which recognizes a peptide epitope in the tandem repeat domain was used at a dilution of 1:3000 and the polyclonal anti-EGFR antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used at a dilution of 1:1000. Antibodies against lamin A/C (Cell Signalling Technology, Beverly MA) and erbB2 (Upstate Biotechnology) were used at dilutions of 1:1000. After washing, blots were incubated with HRP-conjugated species-specific secondary antibodies and immunoreactive bands were visualized using enhanced chemiluminescence (Perkin Elmer, Boston, MA). After detection of the protein of interest, membranes were stripped and reprobed with a polyclonal anti- $\beta$ -actin antibody (Santa Cruz Biotechnology; 1:2000 dilution).

**Cell proliferation assay.** Cells were plated in 24-well plates and siRNA transfections were performed as described above. Cell number was determined at different time points using a CellTiter 96 Assay Kit (Promega, Madison, WI) in quadruplicate assays.

**Cell aggregation assay.** Cells were detached with Versene (Invitrogen) 48 h after siRNA transfection, washed three times with PBS and suspended at a concentration of  $5 \times 10^5$  cells/ml in serum free medium. Cell suspensions were incubated at 37°C in 1% agarose-coated plates with slow agitation (75 rpm) for 45 min and the percentage of cells that formed aggregates was

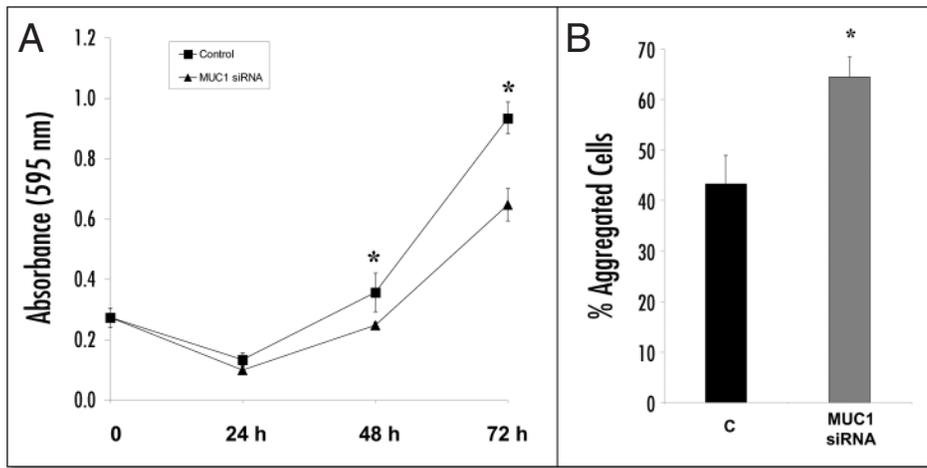


Figure 2. Effect of MUC1 siRNA treatment on cell behavior. (A) KB cells (solid squares) or KB cells treated with MUC1 siRNA (triangles) were assayed at different time points using a commercially available MTT cell proliferation assay. Data from quadruplicate assays were evaluated using a Mann-Whitney U test for statistical significance. \*,  $p < 0.05$ . (B) KB cells transfected with nonsilencing (C) or MUC1 siRNAs were detached 48 h after transfection, replated in 1% agarose-coated plates and incubated at 37°C with gentle agitation for 45 min. The percentage of cells forming aggregates was determined using a hemocytometer and calculated as described in the text. The results of triplicate assays were analyzed for statistical significance using the Mann-Whitney U test. Asterisks indicate significance values of  $p < 0.05$ .

determined using a hemocytometer according to the formula  $([\text{total number of cells}] - [\text{cells remaining as single cells}]) / \text{total number of cells} \times 100\% = \text{percentage aggregation cells}$ . Dead cells were excluded by trypan blue staining and assays were performed in triplicate.

**Soft agar colony formation assay.** Cells ( $10^3$ – $10^4$ ) were mixed with DMEM and 3.3% agarose to a final agarose concentration of 0.3% 24 h after siRNA transfection and aliquots of the cell suspension (1 ml) were added to 12 well plates precoated with 0.5% agarose in DMEM. Colonies were counted 14 days after plating using a Leitz Ortoplan 2 microscope and each assay was performed in triplicate.

**Electrophoretic mobility shift assay.** Electrophoretic mobility shift assays (EMSA) were performed using the DIG Gel Shift Kit (Roche, Indianapolis, IN) according to the manufacturer's instructions. The insert in plasmid pER1-luc (kind gift of Dr. Alfred C. Johnson, NCI, NIH, Bethesda, MD) corresponding to bases -1109 to -16 of the EGFR promoter, was excised with *Hind* III and cleaved with *Bgl* II, *Taq* I, *Sry* I and *Hae* II to yield approx. 200 bp subfragments which were used as templates in the mobility shift assays. Binding reactions were carried out with a recombinant polypeptide containing the MUC1 cytoplasmic tail (MUC1-CT) and purified Sp1 (Promega, Madison WI).

## RESULTS AND DISCUSSION

**MUC1 siRNAs suppress expression of this mucin.** Since MUC1 is expressed at increased levels in many human tumors, previous studies have largely focused on the effects of MUC1 overexpression on cell proliferation and adhesion<sup>23–25</sup> and relatively little is known about the effects of MUC1 downregulation on these properties. In one study, wild-type and Muc1-null mice were crossed with mammary tumor developing mice transgenic for the polyoma virus middle T antigen.<sup>26</sup> Progeny lacking MUC1 displayed a significantly slower growth of mammary gland tumors than mice expressing MUC1.<sup>26</sup> In a more recent study, treatment of YMB-S breast cancer cells with a MUC1 antisense oligonucleotide led to increased cell-cell and cell-surface adhesion, an effect mediated through E-cadherin.<sup>18</sup>

MUC1 is overexpressed in oral epidermoid (squamous cell) carcinomas<sup>27</sup> and we have previously shown that MUC1 is expressed at high levels in KB oral epithelial cells.<sup>21</sup> To develop a model system in which the expression of

MUC1 could be modulated in this cell line, the nucleotide sequence of MUC1 (GenBank accession J05582) was examined for the presence of potential siRNA duplexes and candidate sequences were compared to those in GenBank using a BLAST search. Interfering RNAs corresponding to two sequences displaying no significant homology to other human mRNAs were synthesized and transfected into KB cells. The ability of these two duplexes to suppress MUC1 expression was examined by real time PCR and it was found that MUC1 siRNA 1 (bases 3251–3269) was nearly twice as effective as MUC1 siRNA2 (bases 201–219) in suppressing MUC1 gene expression (data not shown). Levels of suppression with siRNA 1 (used in all subsequent experiments) were approximately 78% relative to levels of MUC1 seen in cells transfected with the nonsuppressing control siRNA at 48 h (Fig. 1A) or 72 h (data not shown). The reduction in MUC1 expression was seen up to 120 hours, which was the last time point examined. When lysates of transfected cells were examined on Western blots using antibodies against the extracellular domain of MUC1, a series of immunoreactive bands ranging from 180 kDa to 250 kDa were observed, likely representing differently glycosylated forms of the extracellular subunit of MUC1. A reduction in MUC1 protein levels was detected in cells transfected with MUC1 siRNA compared to those transfected with the control reagent (Fig. 1B) confirming that mucin protein, as well as mucin mRNA transcripts, were downregulated by treatment with MUC1 duplex siRNAs.

**Role of MUC1 on regulation of cellular behavior.** Previous studies have shown that overexpression of MUC1 leads to profound changes in cell behavior including increased cell proliferation, decreased cell-cell adhesion and increased cell aggregation.<sup>23–25</sup> In the present study, the effect of suppressing MUC1 expression on the proliferation of KB cells was examined in MTT assays where cell growth was monitored at 0, 24, 48 and 72 h after transfection with MUC1 duplex siRNA. At all time points from 24 to 72 h, KB cells transfected with MUC1 siRNA showed decreased growth rates compared with cells transfected with nonsilencing RNA (Fig. 2A). These differences were statistically significant at 48 and 72 h after transfection ( $p < 0.05$ ).

The effect of MUC1 silencing on cell-cell adhesion was then examined in a cell aggregation assay. As shown in Figure 2B, aggregation was significantly increased in cells transfected with MUC1 siRNA compared to cells transfected with the nonsilencing control siRNA. These results are consistent with previous studies indicating that MUC1 plays a significant role in the anti-adhesive properties of cells<sup>18,23–25</sup> and is associated with the metastatic phenotype.

**siRNA-mediated suppression of MUC1 inhibits the expression of EGFR.** Because of the known association of MUC1 and EGFR and the fact that both cell-surface molecules are overexpressed in many cancers, expression of EGFR was examined in MUC1 siRNA treated cells. Somewhat surprisingly, EGFR mRNA levels were significantly reduced (47.8%) in MUC1 knock-down cells, an effect on EGFR expression nearly as great as that seen in cells transfected with EGFR siRNAs (69.7%; Fig. 3A). A reduction in levels of EGFR protein was also seen in MUC1 siRNA treated cells (Fig. 3B) although the extent of reduction was not as pronounced as that seen with EGFR transcript levels. To rule out that EGFR expression levels were modulated by a nonspecific effect of transfection with silencing RNAs, EGFR protein levels were also assayed in cells transfected with an siRNA specific for erbB2 where no significant reduction in EGFR expression was observed (Fig. 3C). Similar results were obtained with the lamin control siRNA provided by the manufacturer (Fig. 3C). Control experiments showed that

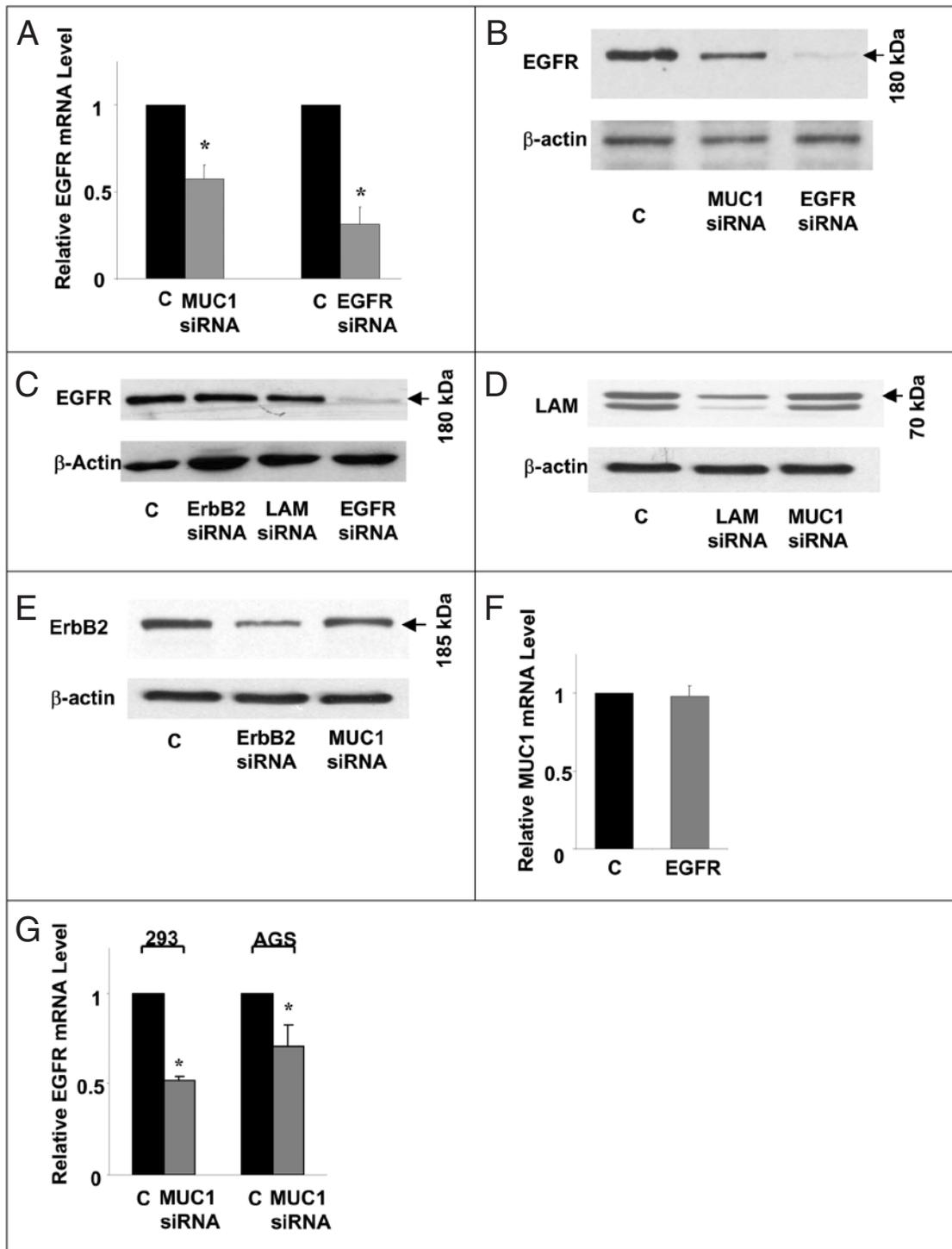


Figure 3. Treatment of cells with MUC1 siRNA downregulates EGFR. (A) KB cells were treated with non-silencing siRNA (C), MUC1 siRNA or EGFR siRNA and EGFR transcript levels were determined by real time PCR and normalized to levels of β-actin transcripts. (B) Lysates from cells treated as in (A) were electrophoresed and blots were probed with an anti-EGFR antibody, stripped and reprobed with an antibody against β-actin. The position of the 180 kDa EGFR protein is indicated with an arrow. (C) KB cells were transfected with non-silencing siRNA (C) or siRNAs for erbB2, lamin A/C (LAM) or EGFR. Proteins in cell lysates were electrophoresed and blots analyzed as described in (B). (D) Lysates from KB cells transfected with nonsilencing siRNA (C) Lamin A/C siRNA were electrophoresed and blots were probed with an anti-lamin A/C antibody, stripped and reprobed with an antibody against β-actin. The position of the 70 kDa lamin doublet is indicated with an arrow. (E) Lysates from KB cells treated with non-silencing siRNA (C), erbB2 siRNA or MUC1 siRNA were electrophoresed and blots were probed with an anti-erbB2 antibody, stripped and reprobed with an antibody against β-actin. The position of the 185 kDa erbB2 is indicated with an arrow. (F) RNA was isolated from KB cells 48 h after transfection with non-silencing RNA (C) or EGFR siRNA and levels of MUC1 transcripts were measured by real-time PCR and normalized to levels of β-actin mRNA. Asterisks indicate significance values of  $p < 0.05$ . G. 293 and AGS cells were transfected with MUC1 siRNA and relative EGFR transcript levels were determined as in (A). Asterisks indicate significance values of  $p < 0.05$ .

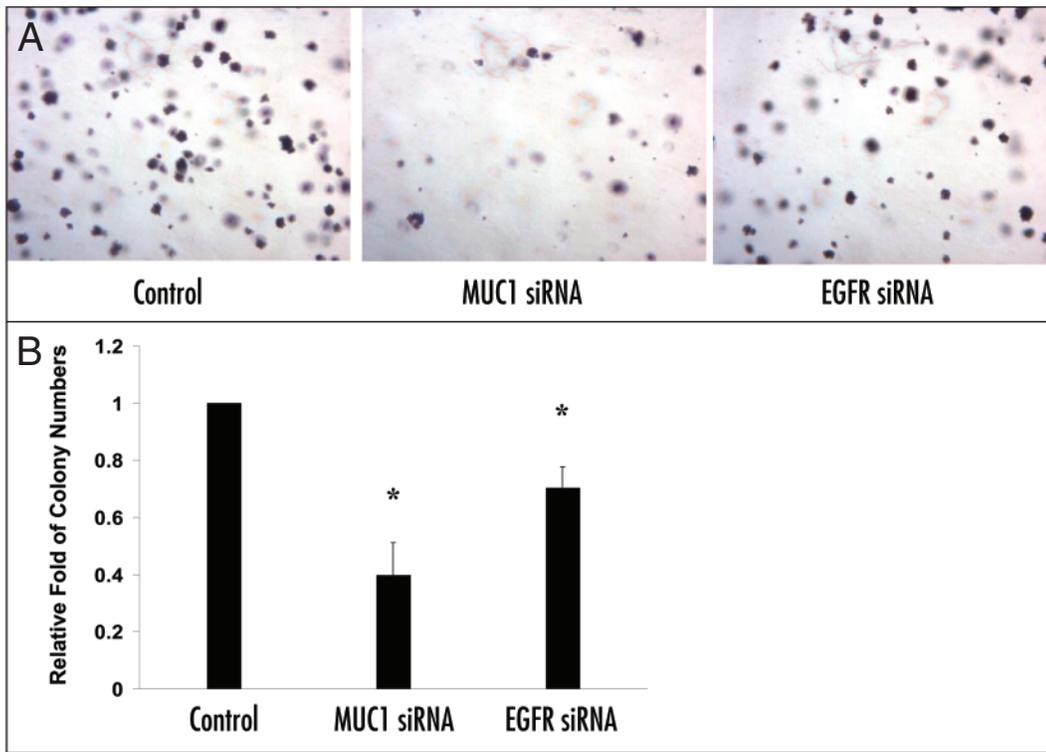


Figure 4. Downregulation of MUC1 dramatically reduces KB cell growth in soft agar. (A) KB cells transfected with non-silencing siRNA (C), MUC1 siRNA or EGFR siRNA were detached 24 h after transfection, mixed with DMEM-3.3% agar to a final agarose concentration of 0.3% and plated on 0.5% agarose-coated plates. Colonies formed in soft agar were counted after 14 days and a representative field from each group is shown. Magnification 2.5 X. (B) Graphical representation of colony numbers from six independent experiments performed in triplicate. Asterisks indicate significance values of  $p < 0.05$ .

levels of lamin A/C and erbB2 protein were significantly reduced in cells treated with the corresponding siRNAs, but not in cells treated with non-silencing or MUC1 siRNAs (Fig. 3D and E). Taken together, these results demonstrate that the observed decrease in EGFR expression was a specific result of MUC1 downregulation. Furthermore, the results show that modulation of the level of another erbB family member did not lead to EGFR downregulation.

oligonucleotides led to a decrease in the ability of treated cells to form colonies in soft agar.<sup>19,20</sup> Similar observations have been made with MUC1.<sup>18</sup> Since we showed that downregulation of MUC1 leads to a parallel decrease in EGFR mRNA and protein levels, studies were conducted to compare the effects of downregulation of both of these molecules on anchorage independent growth in soft agar. Cells treated with EGFR siRNAs displayed a 29.8% reduction in colony formation, whereas cells treated with MUC1 siRNAs displayed nearly double the reduction or 60.3% (Fig. 4A, B). These results show that while both MUC1 and EGFR promote anchorage independent growth in soft agar, downregulation of MUC1 synthesis leads to a much more pronounced growth inhibition, likely due to the additive effects of suppressing both MUC1 and EGFR.

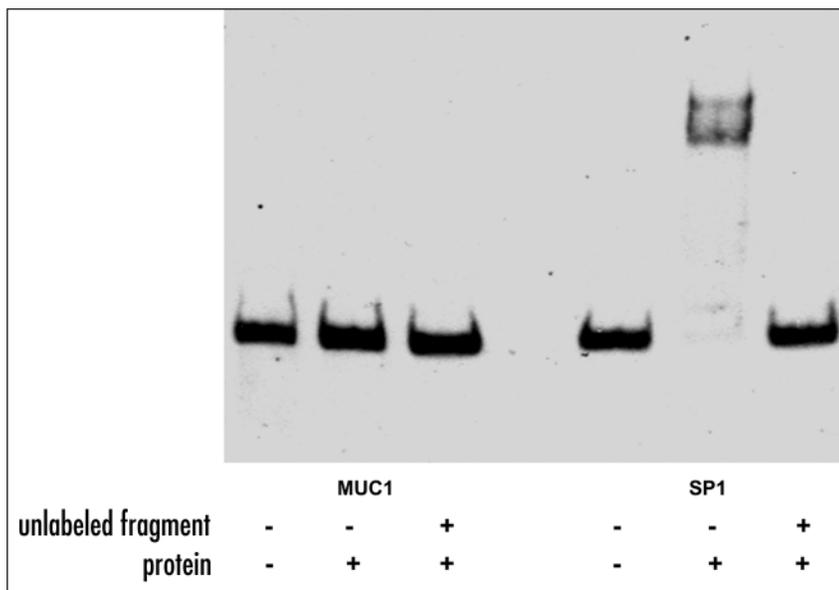


Figure 5. The MUC1 cytoplasmic domain does not bind EGFR promoter fragments. Mobility shift assays were carried out with a digoxigenin labeled EGFR promoter fragment (bases -193 to -16; 0.8 ng) a purified recombinant MUC1 cytoplasmic domain polypeptide (75 ng) and a 125 fold molar excess of unlabeled promoter fragment as indicated. Reaction mixtures were electrophoresed on 5% TBE gels, blotted onto nylon membrane and the blot probed with alkaline phosphatase labeled anti-digoxigenin antibody followed by chemiluminescent detection. Control experiments were carried out with the same template (containing a consensus Sp1 binding site) using purified Sp1 (75 ng). No binding of recombinant MUC1 was observed to any other subfragment of the EGFR promoter (bases -1109 to -16).

We next performed the reciprocal experiment to determine whether treatment of cells with EGFR siRNA would lead to a parallel decrease in MUC1 expression. RNA was isolated from cells transfected with either the non-silencing control siRNA or EGFR siRNA and MUC1 transcript levels were determined by real-time PCR. No difference in MUC1 expression levels was detected (Fig. 3F) providing further evidence that the effect of MUC1 downregulation on EGFR expression is specific.

In order to determine if the observed effect of MUC1 downregulation on EGFR expression was specific to KB cells, two additional cell lines, 293 human embryonic kidney cells which express low levels of MUC1, and AGS human gastric adenocarcinoma cells, which express moderate levels of MUC1, were treated with MUC1 siRNA. EGFR transcript levels were reduced by 49.4% and 39% in 293 and AGS cells, respectively, comparable to the level of reduction seen initially in KB cells (Fig. 3G).

**Reduction of MUC1 inhibits colony formation.** It has previously been reported that downregulation of EGFR mRNA by antisense

Several different mechanisms could explain the apparent regulation of EGFR expression by MUC1. MUC1 could act directly, binding to one or more cis acting elements in the EGFR promoter leading to transcriptional control. This seemed like a reasonable possibility since a fragment of the MUC1 cytoplasmic tail has been shown to translocate to the nucleus<sup>8-9</sup> and to be involved in the regulation of expression of cyclin D1 and possibly other Wnt target genes.<sup>10</sup> However, in gel shift assays, we found no binding of a recombinant MUC1 cytoplasmic domain polypeptide to any site in the proximal 1 kb of the EGFR promoter (Fig. 5) although the possibility remains that binding of MUC1 to one or more upstream sites could regulate transcription. If MUC1 does not regulate EGFR transcription directly, it could bind to other protein factors involved in EGFR transcriptional regulation and either potentiate or block the effects of these proteins. Alternatively, MUC1 could act through a post-transcriptional mechanism affecting the stability or turnover rate of EGFR transcripts.

The present results have identified a novel role for MUC1 in the progression of cancer. While many of the phenotypic changes associated with malignant cells result from overexpression of aberrantly glycosylated MUC1, we have found that MUC1 can also modulate the behavior of malignant cells by regulating the expression of the EGFR receptor tyrosine kinase. While MUC1 and EGFR are coordinately overexpressed in many breast and oral squamous cell carcinomas,<sup>15-17,27,28</sup> their expression patterns do not correlate in other malignancies such as nonsmall cell lung cancer.<sup>29</sup> This suggests that the MUC1 regulatory effect on EGFR may be tissue and/or cell type specific and represents only one mechanism by which EGFR expression is regulated. A complete understanding of this regulatory mechanism could lead to the development of novel therapeutic modalities to down regulate expression of both MUC1 and EGFR.

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