Functions of MUC16 in Corneal Epithelial Cells

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Purpose. The membrane-associated mucin MUC16, a heavily O-glycosylated transmembrane protein, is expressed by the ocular surface epithelia and localized on the tips of the surface microplicae. Although its functions in the ocular surface glycocalyx are unknown, it is thought that MUC16 provides a disadhesive barrier to the epithelial membrane. Two other membrane-associated mucins expressed by ocular surface epithelia, MUC1 and MUC4, are multifunctional and have signaling capabilities through their cytoplasmic tails and EGF-like domains, respectively. The MUC16 cytoplasmic tail has not been characterized, but, because it contains a polybasic amino acid sequence, it potentially interacts with the actin cytoskeleton through ezrin/radixin/moesin (ERM) actin-binding proteins.

Methods. The interaction of MUC16 with the actin cytoskeleton through ERMs was investigated using cytoplasmic tail peptides and ERM pull-down experiments. MUC16 functions were determined using RNA interference in immortalized human corneal-limbal epithelial (HCLE) cells. The effect of MUC16 knockdown on microplicae structure in HCLE cells was determined using scanning and immunoelectron microscopy. HCLE cells were incubated with rose bengal dye to measure the role of MUC16 in ocular surface barrier function. Binding of fluorescently labeled *Staphylococcus aureus* to HCLE cells was measured to determine the role of MUC16 in the protection of pathogen adherence on the ocular surface epithelium.

Results. MUC16 cytoplasmic tail peptides bound the N-terminus of ERMs, with no detectable binding of MUC1 and MUC4 peptides. No effect on surface membrane projections could be detected in HCLE cells after MUC16 suppression; however, HCLE cells incubated with rose bengal showed that exclusion of the dye was significantly reduced in cells with MUC16 suppression. In addition, *S. aureus* binding to HCLE cells was significantly increased with MUC16 suppression.

Conclusions. These results suggest that MUC16 is a multifunctional molecule linked to the actin cytoskeleton. The expression of MUC16 in the ocular surface glycocalyx helps provide a disadhesive protective barrier for the epithelial surface. (*Invest Ophthalmol Vis Sci.* 2007;48:4509–4518) DOI:10.1167/iovs.07-0430

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Mucins are a group of at least 20 glycoproteins of high molecular weight that contain tandem repeats of amino acids rich in serine and threonine and that serve as sites for O-glycosylation. Up to 80% of the mass of the mucin molecule can be made up of O-glycans.[1] Two types of mucins are known, secreted and membrane associated; both are present on the apical side of all wet-surfaced epithelia.[2] Secreted mucins are produced by specialized goblet cells within the wet-surfaced epithelia and acini of epithelial glands.[3] Membrane-associated mucins are components of the glycocalyx of the wet-surfaced epithelia, where they may extend 200 to 500 nm from the apical luminal surface.[4,5]

In humans, 10 membrane-associated mucin genes are known (MUC1, MUC3A, MUC3B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17, MUC20), and most wet-surfaced epithelia express several, albeit in different combinations and ratios.[6,7] At the ocular surface, at least three membrane-associated mucins, MUC1, MUC4, and MUC16, were expressed.[3] All membrane-associated mucins have a single transmembrane-spanning domain, a short cytoplasmic tail, and a large, heavily glycosylated extracellular domain. Cytoplasmic tail and extracellular domain sequences of the membrane-associated mucins are highly variable and may impart unique functions to each mucin. Based on data from MUC1 and MUC4, the membrane-spanning mucins are hypothesized to be multifunctional molecules in that their extracellular domains form the glycocalyx that protects, hydrates, and lubricates the apical epithelial surface, while the cytoplasmic tail of MUC1 and the EGF-like domains in the extracellular domain of MUC4 may participate in cell signaling or may bind to intracellular molecules.[5,8,9]

MUC16, previously known as CA125, is the largest membrane-associated mucin cloned to date and is well known as a serum marker for ovarian cancer.[10] Even though CA125 has been studied for many years, because of its large size (22,000 amino acids) and its extensive glycosylation, the entire gene has only recently been cloned.[10–12] MUC16 is also expressed on the apical surfaces of noncancerous native epithelia such as those on the ocular surface, respiratory tract, and female reproductive tract.[6,13–16] Expression of MUC16 is altered on the conjunctival epithelium of patients with non-Sjögren dry eye syndrome, as shown by H185 antibody, an antibody that recognizes a carbohydrate epitope on MUC16.[17] Although it is hypothesized that MUC16, in combination with the other membrane-associated mucins, forms the glycocalyx, which provides a disadhesive surface barrier[18] to protect the epithelium from large molecule or pathogen penetration and which maintains its hydration, direct evidence is not available, nor are there data regarding the specific functions of MUC16. Furthermore, the characteristics, binding, and signaling capabilities of the MUC16 cytoplasmic tail are unknown.

The ezrin/radixin/moesin (ERM) family, which is composed of ezrin, radixin, moesin, and merlin, is a family of ubiquitous proteins with high amino acid homology.[19] They are colocalized with actin filaments beneath the cell membrane of specialized extensions on the apical surfaces of epithelial cells, such as microvilli, filopodia, and membrane ruffles.[20–23] Ezrin knockout mice show a significant reduction in the formation of apical microplicae.[22] The ERM proteins bind F-actin through their carboxyl termini, and, through their amino termini, they

bind polybasic sequences in the cytoplasmic tails of membrane-bound proteins such as CD44 and intracellular adhesion molecule 2 (ICAM-2), thus linking membrane-spanning molecules to the actin cytoskeleton.[24–27] MUC16 contains a polybasic sequence of amino acids in its cytoplasmic tail similar to ICAM-2 and CD44, which suggests that it may interact with ERMs in a similar manner. Direct evidence of ERM protein binding to the cytoplasmic tails of membrane-associated mucins has not been shown.

Knockdown methods to determine the specific functions of two other membrane-associated mucins, MUC1 and MUC4, have been reported. MUC1 knockout in mice results in varied phenotypes, including reduced intestinal uptake of cholesterol, increased susceptibility to ocular surface infection, and enhanced pulmonary clearance of *Pseudomonas*.[28–30] Expression of MUC1 has also been knocked down using RNA interference (RNAi) in several cell lines, inhibiting cell proliferation and colony formation by epithelial carcinoma cells[31] and adhesion and tumor formation by human pancreatic cells.[32] In addition, MUC1 knockdown results in internalization and degradation of ErbB1 in breast epithelial cells, which suggests that MUC1 is a binding partner for ErbB1.[33] MUC4 expression has also been suppressed using antisense RNA in a pancreatic tumor cell line, resulting in decreased cell growth and tumor formation and increased cell adhesion and aggregation.[34] To our knowledge, the effects of the targeted knockdown of MUC16 expression to study its specific functions have not been reported.

The purpose of this study was to determine the specific functions and intracellular interactions of MUC16 on the apical cell surface of corneal epithelial cells and to investigate the potential multifunctional properties of MUC16. Transfecting full-length MUC16 into cells to study specific function is not practical because of its extraordinary size. Therefore, we used RNA interference methods to stably knock down MUC16 expression in an immortalized human corneal limbal epithelial (HCLE) cell line, a model for differentiated wet-surfaced stratified epithelium optimized to express membrane-associated mucins. HCLE cells express high levels of MUC16 in addition to the membrane-associated mucins MUC1 and MUC4.[35] We studied the interaction of MUC16 and ERMs and the effect of MUC16 knockdown on barrier protection, pathogen adherence, and apical cell surface architecture.

METHODS

Tissue Collection

All tissue was obtained in accordance with good clinical practice, Institutional Review Board and informed consent regulations of the Schepens Eye Research Institute and the Massachusetts Eye and Ear Infirmary, and the tenets of the Declaration of Helsinki. Corneal tissue from cadavers was obtained from the Lions Eye Bank, flash frozen, and processed for immunoelectron microscopy.

Cell Culture

Immortalized human corneal limbal epithelial (HCLE) cells were maintained at 37°C at 5% CO_2 and grown as previously reported to optimize mucin expression.[35] Briefly, HCLE cultures were grown in keratinocyte serum-free medium (K-SFM; Invitrogen, Carlsbad, CA) to confluence. After reaching confluence, cells were switched to DMEM/F12 supplemented with 10% calf serum and 10 ng/mL epidermal growth factor (EGF) for 7 days, which promotes stratification, differentiation, and mucin gene expression.[35]

ERM Expression in Epithelia

Total RNA was isolated from HCLE cell cultures using reagent (TRIzol; Invitrogen), as previously described.[13] After elimination of any residual genomic contamination by digestion with amplification grade DNase I (Invitrogen), total RNA was reverse transcribed (1 μg in 20 μL) using random hexamer primers and reverse transcriptase (Superscript II; Invitrogen), as previously described. Conventional RT-PCR was performed to confirm the presence of message for ERM using published primers.[36] ERM protein expression was confirmed by immunoblot of SDS-PAGE–separated proteins from HCLE cell cultures using anti–ERM C-19 antibody (reacts with ERM [Santa Cruz Biotechnology, Santa Cruz, CA]) and chicken anti-merlin.[36]

TABLE 1. Peptides Synthesized for Pull-Down Experiments

Mucin	Peptide Sequence
MUC16 CT	TTRRRKKEGEYNVQQQCPGYYQSHLDLEDLQ
MUC16 control	TTQIELAEGEYNVQQQCPGYYQSHLDLEDLQ
MUC1 CT	RRKNYGQLDIFPARDYHPMCEYPTYHTHGRY
MUC1 control	IQENYGQLDIFPARDYHPMCEYPTYHTHGRY
MUC4 CT	LRFWGCSGARFSYFLNSAEALP

Basic amino acids (and their replacements) are underlined.

Pull-Down Assays

Peptides corresponding to the cytoplasmic tail of MUC1, MUC4, and MUC16 were synthesized by solid-phase procedure, using F-moc chemistry, in the Peptide Synthesis Core of the Reproductive Endocrine Sciences Center (Massachusetts General Hospital, Boston, MA). As additional controls, peptides were synthesized corresponding to the cytoplasmic tails of MUC1 and MUC16 with the basic amino acids replaced by nonbasic residues (Table 1).

Equal amounts of the peptides were immobilized on agarose beads (Sulfo-Link Kit; Pierce Rockford, IL) in accordance with the manufacturer's recommendations. Nonspecific binding sites on the agarose beads were blocked by incubation with L-cysteine-HCl. The agarose-immobilized peptides were then used in pull-down experiments with glutathione S-transferase (GST)-fusion proteins of the N-terminal (amino acids 1–332) or C-terminal (amino acids 305–577) portions of moesin recombinantly expressed as previously described.[36] Equal amounts of GST-N-terminal moesin, GST-C-terminal moesin (as negative control), and GST alone (as control for nonspecific binding to GST) were incubated with equal amounts of immobilized peptide on a rocker overnight at 4°C. The beads were collected by centrifugation, washed twice in PBS with 0.05% NP-40 plus protease inhibitor (PI), and washed three times in PBS plus PI. Bound protein was eluted from the centrifuged beads by incubation in Laemmli sample buffer. Proteins were separated by SDS-PAGE (4% stacking, 10% separating), and gels were stained with Coomassie blue. The presence of a band of appropriate molecular weight was indicative of binding of the fusion protein to the immobilized peptide. GST-fusion proteins have different molecular weights, as follows: N-terminal fusion protein, 65.7 kDa; C-terminal fusion protein, 58.8 kDa; GST, 27.5 kDa.

siRNA Design and Transfection

Three potential sequences targeting the human *MUC16* gene were chosen using software from OligoEngine, Inc. (Seattle, WA). Custom complementary oligonucleotides were synthesized, annealed, and ligated into a mammalian expression vector (pSuperRetro-puro; OligoEngine, Inc., Seattle, WA) that directs the intracellular synthesis of siRNA transcripts.[37] Insert sequences used were: Sequence #1, sense, 5′-AGCCACCTCATCTATTACCTTCAAGAGAGGTAATAGATGAGGTGGCT-3′, antisense, 5′-AGCCACCTCATCTATTACCTCTCTTAGAAGGTAATAGATGAGGTGGCT-3′; Sequence #2, sense, 5′-CTGCATGTACTCCCATCTCTTCAAGAGAGAGATGGGAGTAGATGCAG-3′, antisense, 5′-CTGCATCTACTCCCATCTCTCTCTTGAAGAGATGGGAGTAGATGCAG-3′; Sequence #3, sense, 5′-TAACCATCACCACCCAAACTTCAAGAGAGTTTGGGTGGTGATGGTTA-3′, antisense, 5′-TAACCATCACCACCCAAACTCTCTTGAAGTTTGGGTGGTGATGGTTA-3′. The ligated vectors were transformed into chemically competent *Escherichia coli* (DH5α cells), correct orientation of the

hairpin siRNA insert was verified with DNA sequencing, and large-scale cultures were grown to obtain a sufficient amount of plasmid vector. Using lipid-mediated transfection reagents, the vectors were transfected into 293–10A1 cells, a packaging cell line that produces high-titer retrovirus in culture (ATCC). HCLE cells were then seeded in 24-well plates at 2×10^4 cells per well and grown to 40% confluence in serum-free growth medium. Tissue culture medium from transfected 293–10A1 cells[38] was filtered 48 hours after transfection, and the viral supernatant was used to infect cultures of the subconfluent HCLE cells after the addition of 4 μg/mL polybrene. Isolated clones were obtained using antibiotic selection (puromycin) and further expanded to confluence to obtain stably transfected cells. Then the transfected cells were grown to confluence and switched to stratification medium as described.

Real-Time Polymerase Chain Reaction

Total RNA was extracted from cultures using reagent (TRIzol; Invitrogen) according to the manufacturer's instructions. First-strand cDNA was synthesized according to the manufacturer's protocol from 1 μg DNase-treated RNA with a combination of oligo dT and random hexamer primers using reverse transcriptase (iScript; Bio-Rad, Hercules, CA) followed by RNase H treatment (Invitrogen).

cDNA was generated from cultures as described. Relative expression of MUC1 and MUC16 was determined using real-time quantitative RT-PCR performed as previously described on a sequence detection system (ABI Prism 7900HT; Applied Biosystems, Foster City, CA)[39] using TaqMan, with calculations based on the ΔCt method.[39] Primers and probes for detection of MUC1, MUC4, and MUC16 were reported previously,[39] and GAPDH primers and probes were purchased from Applied Biosystems. Relative levels of mucin mRNA were calculated using the nontransfected control as the calibrator, and they were normalized to GAPDH expression. Statistical comparisons of the real-time RT-PCR results were performed using the Fisher protected least-significant difference (PLSD) test (Statview 5.0 for MacIntosh; SAS Institute, Inc. Cary, NC). $P < 0.01$ was considered significant.

Western Blot Analysis

Protein was extracted using 2% sodium dodecyl sulfate (SDS) plus protease inhibitor cocktail (Pierce) and separated on 1% agarose gels, followed by transfer to nitrocellulose with vacuum blotting (25 μg per lane). Immunoblots were performed using an antibody specific to MUC16 (OC125), as previously described.[39] Quantification of MUC16 protein knockdown was determined by densitometry of MUC16 immunoblots.

Dye Penetrance Assay

Stably transfected HCLE cells expressing MUC16 siRNA were grown to confluence in 24-well plates in K-SFM, followed by incubation in stratification medium for 7 days to induce mucin expression. Nontransfected cells and cells transfected with empty vector were used as negative controls. Culture medium was aspirated, and cells were washed three times with phosphate-buffered saline (PBS), followed by a 5-minute incubation of 0.1% rose bengal dye in calcium and magnesium-free PBS. The dye solution was removed, and cultures were photographed as previously described.[40] Cells were photographed at preconfluence and confluence and after 7 days in stratification medium. The area of islands of stratified cells that excluded rose bengal was quantified in culture images using ImageJ analysis software.[41]

Bacterial Adherence Assay

MUC16 knockdown cells, nontransfected cells, and vector-transfected HCLE cells were grown to confluence in K-SFM and incubated in stratification medium for 7 days in four-well chamber slides. *Staphylococcus aureus* ALC 1435 was labeled with FITC (0.1 mg/mL in phosphate-buffered saline) for 30 minutes followed by two washes in PBS. Then HCLE cells were washed with DMEM-F12 and incubated with 2.2×10^6 cells per well of FITC-labeled *S. aureus* ALC1435 for 1 hour at 37°C. Cells were washed three times with PBS and fixed in 4% paraformaldehyde. Immunofluorescence microscopy was performed using antibodies to MUC1 (HMFG-2) or MUC16 (OC125), and nuclei were labeled with DAPI. The amount of adherent bacteria was quantified using ImageJ analysis software.

Immunoelectron Microscopy

Pre-embedding immunolocalization of monoclonal mouse anti–human MUC16 antibody (OC125; Dako, Carpinteria, CA) with 12-nm colloidal gold conjugated donkey anti–mouse IgG (Jackson ImmunoResearch, West Grove, PA) was performed on stratified HCLE cells grown on tissue culture slides. Cells were fixed in 2% paraformaldehyde and scraped from the slides. Cell sheets were then incubated in primary and secondary antibodies overnight at 4°C at dilutions of 1:25. After labeling, specimens were fixed in half-strength Karnovsky fixative, followed by incubation in 2% OsO_4, then dehydrated in a series of ethanols and propylene oxides and embedded in Epon/Araldite for transmission electron microscopy.

MUC16 was also localized by postembedding immunoelectron microscopy using the monoclonal antibody H185, which recognizes a carbohydrate epitope on MUC16,[13] on stratified HCLE cells grown as described and fixed with 0.2% glutaraldehyde and 4% paraformaldehyde, as previously described[42] using goat anti-mouse IgG secondary antibody conjugated to 10-nm colloidal gold (Ted Pella, Redding, CA).

Double labeling of MUC16 and ERMs was performed on frozen pieces of human cornea (approximately 2 mm cut by hand). MUC16 was localized using pre-embedding techniques, described here, in addition to pre-embedding immunolocalization of polyclonal goat anti–ERM binding (C-19; Santa Cruz;) with 6-nm colloidal gold-conjugated donkey anti– goat IgG (Jackson ImmunoResearch). After tissue fixation in 2% paraformaldehyde, the sections were permeabilized with 0.3% Triton-X-100 for 20 minutes. The sections were then incubated in primary and secondary antibodies as described and processed for transmission electron microscopy.

Scanning Electron Microscopy

Immunolabeling of MUC16 for scanning electron microscopy was also performed on stratified HCLE cultures grown on 12-mm round coverslips in 24-well culture plates using monoclonal mouse anti–human MUC16 (OC125). Cells were fixed in 2% paraformaldehyde, rinsed, and labeled with OC125 antibody and then with 6-nm colloidal gold-conjugated donkey anti–mouse IgG (Jackson ImmunoResearch). Each labeling was performed for 24 hours at 4°C. After labeling, cultures were fixed for 24 to 48 hours in half-strength Karnovsky fixative, rinsed three times in PBS, dehydrated in a series of ethanols, critical point dried in a critical point dryer (Samdri-795; Tousimis, Rockville, MD) and coated with a 150A carbon layer (Ion Beam Coater; Gatan, Pleasanton, CA). Cells were then viewed on a scanning electron microscope (FESEM 7401F; Field-Emission Scanning Electron Microscope; JEOL, Peabody, MA) using backscatter detection to visualize the gold labeling.

Results

Telomerase-transformed HCLE cells were chosen to assess the functions of MUC16. These cells express high levels of MUC16, making them an ideal model to test its functions in native ocular surface epithelial cells. HCLE cells, which stratify and differentiate in vitro over time, are particularly useful in studying the roles of MUC16 in glycocalyx and membrane surface architecture, pathogen adherence, and barrier function (dye penetrance).[35]

Interaction of MUC16 with ERMs

Given that MUC16 is highly expressed on the tips of microplicae of the ocular surface epithelium, the potential interaction of the MUC16 cytoplasmic tail with the ERM family of proteins

was investigated because of its role in actin cytoskeleton organization and microvilli formation.[21] The interaction of the MUC16 cytoplasmic tail with members of the ERM family of proteins and its role in the formation of apical epithelial membrane architecture was examined because of the presence of a polybasic sequence of amino acids (RRRKK) in its cytoplasmic tail sequence located near the membrane-spanning domain. Expression of ERM and merlin message (data not shown) and protein (Fig. 1A) was verified in cultures of stratified HCLE cells. Ezrin and radixin have similar molecular weights and comigrate at approximately 80 kDa, whereas moesin migrates at approximately 75 kDa. To assess the interaction of the cytoplasmic tail of MUC16 with moesin (one of the ERM family members), pull-down experiments were performed using synthetic peptides mimicking the cytoplasmic tail sequence and constructs of the N-terminus of moesin recombinantly expressed and fused with GST, with a C-terminal moesin construct as a control. For comparison, peptides were synthesized containing the cytoplasmic tail sequences of the two other membrane-associated mucins expressed by HCLE cells, MUC1 and MUC4 (Table 1). The cytoplasmic tail peptides were synthesized corresponding to the cytoplasmic tails of MUC1 and MUC16 containing either the wild-type polybasic sequence of amino acids (+B) or a nonbasic control sequence (−B). Given that MUC4 has no polybasic sequence in its cytoplasmic tail, a nonbasic control sequence was not synthesized. The cytoplasmic tail peptides were immobilized on agarose beads and incubated with the GST-tagged moesin fusion proteins, and eluted protein was separated on an SDS-PAGE gel. The pull-down experiments showed that the N-terminal moesin peptide bound to the cytoplasmic tail peptide of MUC16 (Fig. 1B) but not to the MUC16 peptide lacking the polybasic sequence or to the GST negative controls. Binding of the C-terminal portion of moesin to the MUC16 cytoplasmic tail peptide was not observed. Binding of moesin to MUC1 and MUC4 peptides were not observed, but weak, nonspecific binding was evident between the MUC1 sequence lacking the polybasic sequence and the N-terminal moesin construct.

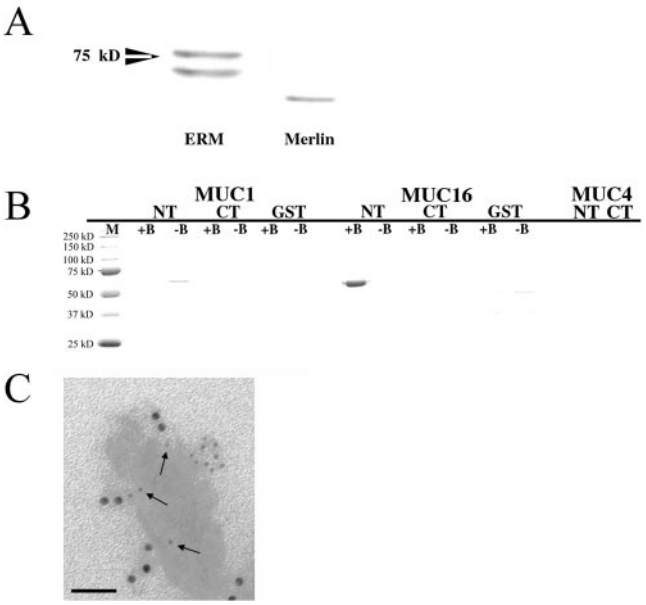

FIGURE 1. Interaction of MUC16 with ERM proteins. (**A**) Expression of ERM (*left*) and merlin (*right*) proteins in HCLE cells by immunoblot. Ezrin and radixin comigrate at 80 kDa, whereas moesin migrates at 75 kDa. (**B**) Coomassie blue–stained SDS-PAGE gel of protein eluted from mucin cytoplasmic tail peptide-immobilized agarose beads bound with GST-tagged N-terminal (NT) and C-terminal (CT) moesin constructs (GST, negative control; M, molecular weight markers). (**C**) Immunoelectron microscopy of fixed human cornea sections showing colocalization of MUC16 (large gold particles) and ezrin (small gold particles, *arrowheads*) on the microplicae. Scale bar, 0.1 μm.

The apical cell membrane of the ocular surface epithelia has surface membrane folds known as microplicae, into which actin filaments insert.[43] Because the MUC16 cytoplasmic tail peptides and the N-terminal moesin construct interact in vitro, MUC16 and ERMs were localized in epithelial cells using double-labeling immunoelectron microscopy on fixed sections of human cornea (Fig. 1C). These sections were simultaneously incubated with OC125, a monoclonal antibody to MUC16, and a pan-ERM antibody, with a gold-conjugated secondary antibody specific to each. MUC16 (larger gold particles) was localized on the apical surface of the microplicae with a 12-nm gold-conjugated secondary antibody. ERMs (smaller gold particles) were localized to the cytoplasmic face of the microplicae cell membrane proximal to MUC16 using a 6-nm gold-conjugated secondary antibody. This result supports the pull-down data that ERMs bind to the cytoplasmic tail of MUC16, potentially linking it to the actin cytoskeleton, which is known to be present within these structures.

Knockdown of MUC16 Expression in HCLE Cells

Stably transfected clones of HCLE cells were generated expressing three different MUC16 siRNA sequences. Real-time PCR analysis was performed on all cell lines to determine the amount of MUC16 mRNA expression knockdown. Of the three siRNA sequences tested in HCLE cells, sequences 1 and 2 showed 77% and 81% knockdown of MUC16 mRNA expression, respectively, compared with nontransfected and vector-transfected controls (Fig. 2A; $n = 3$; $P < 0.01$). siRNA sequence 3 was not effective at reducing MUC16 expression. MUC16 knockdown in HCLE cells did not significantly affect MUC1 and MUC4 mRNA expression, the two other membrane-associated mucins expressed by the cells.

MUC16 protein levels were also significantly reduced with stable transfection of siRNA sequences 1 and 2 (Fig. 2B; $n = 3$). Densitometry of immunoblots indicated that protein expression was reduced by 89% and 94% with sequence 1 and 2, respectively (Fig. 2C; $n = 3$; $P < 0.01$).

To determine whether MUC16 protein knockdown resulted in reduced levels within the apical membranes, immunofluorescence microscopy was performed to localize expression of MUC16 in nonpermeabilized HCLE cells (Figs. 3A–D). Figures 3A and 3B show the amount of cell surface expression of MUC16 in nontransfected and vector-transfected HCLE cells, respectively. Less binding is observed in HCLE cells expressing siRNA sequences 1 and 2 (Figs. 3C and 3D, respectively), though some binding is still evident. This is most likely because the antibody used for MUC16 immunolocalization (OC125) binds to the tandem repeat region of MUC16 and, thus, will bind to each molecule multiple times,[13] enhancing the signal on the remaining MUC16 in the knockdown cell lines. We found no alteration in cell appearance or time required to reach confluence on knockdown of MUC16 in HCLE cells.

Effect of MUC16 Suppression on HCLE Cell Surface Architecture

Microplicae, which are present on the ocular surface epithelium, are observed in the differentiated HCLE cell line (Fig. 4). MUC16 is localized to the tips of microplicae on the apical cells of the native and cultured corneal epithelium.[3] In this study, two antibodies recognizing MUC16 were used to localize expression in stratified HCLE cells. H185 antibody, which recognizes an O-acetylated sialic acid epitope on MUC16,[40] is localized on the microplicae and in cytoplasmic vesicles (Fig. 4A).

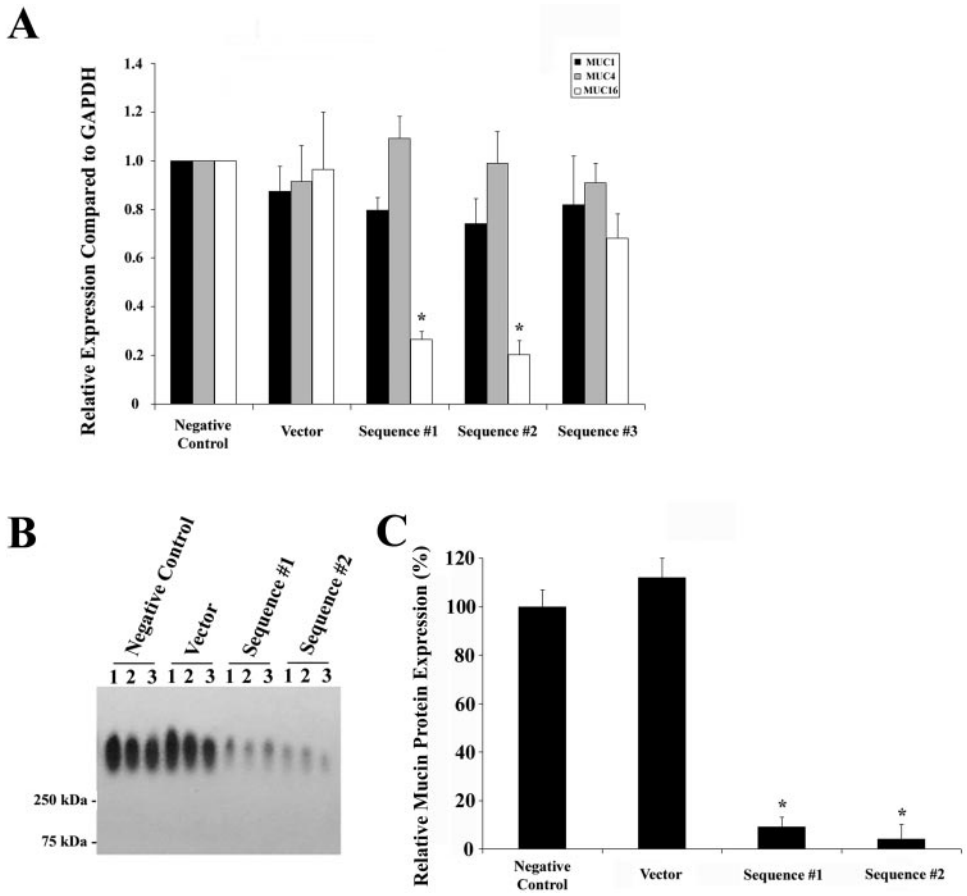

FIGURE 2. Expression of MUC16 in stably transfected HCLE cells. (**A**) Real-time PCR detection of MUC1, MUC4, and MUC16 message in stably transfected HCLE cells. Results were normalized to GAPDH expression and expressed relative to the nontransfected control ($n = 3$; $^*P < 0.01$). (**B**) Representative immunoblot showing knockdown of MUC16 protein in HCLE cells with MUC16 siRNA sequences 1 and 2. MUC16 protein was detected by immunoblots from agarose gels using the OC125 antibody. (**C**) Quantification of MUC16 protein knockdown by densitometry of MUC16 immunoblots ($n = 3$; $^*P < 0.01$). Error bars, SEM.

The OC125 antibody, which recognizes the tandem repeat region in the extracellular domain of MUC16, is also localized on the tips of the microplicae (Fig. 4B).

MUC16 localization on the tips of microplicae on the apical cells of the corneal epithelium and the pull-down data showing the MUC16 cytoplasmic tail association with the N-terminal domain of moesin led to the hypothesis that the mucin may be involved in the formation of these structures. Field-emission scanning electron microscopy revealed that the presence of microplicae on the surface of HCLE cells was not altered in the

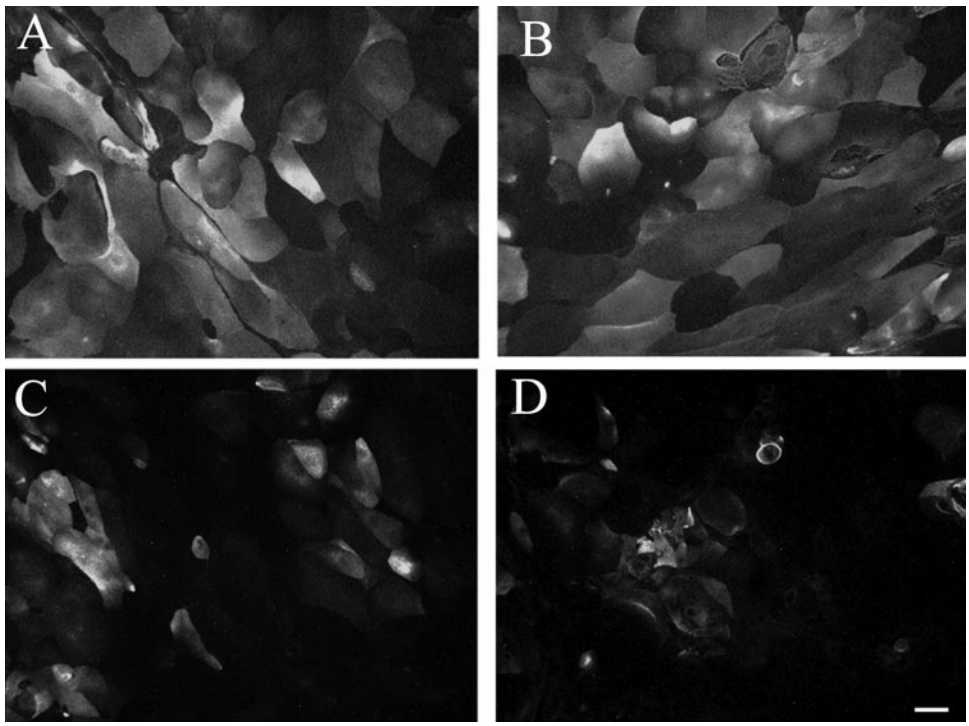

FIGURE 3. Microscopy of HCLE cells with MUC16 expression knockdown. (**A–D**) Immunofluorescence microscopy showing localization of MUC16 expression in nonpermeabilized HCLE cells that were (**A**) nontransfected, (**B**) vector-transfected, (**C**) MUC16 siRNA sequence 1-transfected, and (**D**) MUC16 siRNA sequence 2-transfected. Scale bar (**A–D**), 50 μm.

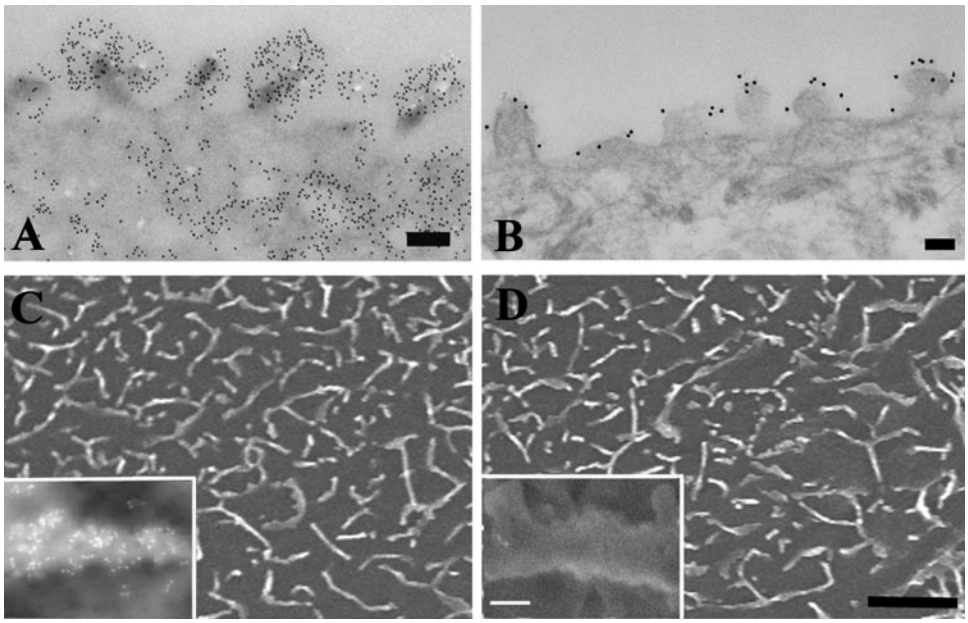

FIGURE 4. Localization of MUC16 on human corneal epithelial cell cultures. (**A**) Localization of H185 epitope (which recognizes a carbohydrate epitope on MUC16) in stratified HCLE cells. Gold particles, 10 nm. (**B**) Localization of MUC16 protein using the OC125 antibody in stratified HCLE cell cultures. Gold particles, 12 nm. (**C**, **D**) Scanning electron microscopy of the surface of (**C**) nontransfected and (**D**) MUC16 siRNA sequence 2-transfected HCLE cells. (**C**, **D**) Field emission scanning electron microscopy showing localization of MUC16 on microplicae (*insets*). Scale bar: (**A**, **B**) 0.4 μm; (**C**, **D**) 1 μm; (**C**, **D** *insets*) 0.1 μm.

knockdown cell lines (Figs. 4C, 4D). Higher magnification showed that MUC16 was reduced on the microplicae of the knockdown cells (Figs. 4C, 4D, insets).

Effect of MUC16 Suppression on Dye Penetrance in HCLE Cells

Rose bengal, an anionic dye used to assess damage to the ocular surface epithelium,[44] was previously shown to be excluded by differentiated islands of stratified HCLE cells that express MUC16.[45] To determine whether MUC16 was responsible for preventing dye penetrance, we examined the uptake of rose bengal in control and MUC16 knockdown cell lines cultured to subconfluence, confluence, and 7 days after confluence when cells are stratified. In subconfluent HCLE cells, which express no membrane-associated mucins, and in confluent cells, which express MUC1,[45] dye penetrance into the cytoplasm and nucleus was observed (Fig. 5A). When the cells were placed in stratification medium for 7 days to induce the expression of MUC1, MUC4, and MUC16,[35] islands of stratified cells that excluded the rose bengal dye were evident. Significantly fewer islands excluding the dye were seen in stratified HCLE cells in which MUC16 expression was suppressed. Areas of islands were quantified (Fig. 5B), showing that cells expressing siRNA sequence 1 had 64% less island area per field, whereas sequence 2 had 83% less ($n = 5$; $P < 0.01$). These data suggest that cells with reduced MUC16 expression lack the dye penetrance barrier observed in control stratified HCLE cells.

Effect of MUC16 Suppression on *Staphylococcus aureus* Binding in HCLE Cells

The glycocalyx is important in protecting the epithelial surface from pathogen adherence and penetration.[46] For example, bacterial adherence is not observed in intact, unwounded native ocular surface epithelia.[46,47] It has been suggested that membrane-associated mucins play a role in protecting the apical surface from pathogen adhesion and possible penetration.[48] Because MUC16 is an exceptionally large and prevalent membrane-associated mucin in the glycocalyx of the corneal epithelium,[13] we hypothesize that its presence on the apical membrane helped to prevent pathogen adherence by forming a barrier on the epithelial surface.

S. aureus is a known pathogen at the ocular surface epithelium[49] and is commonly used to assess host–pathogen interactions. Thus, we measured the effect of MUC16 suppression in stratified HCLE cells on binding of *S. aureus* to the cell surface. Stratified HCLE cells were incubated with a GFP-producing strain of *S. aureus* (also labeled with FITC), washed, fixed, and then observed by immunofluorescence microscopy to localize MUC16. Higher numbers of bound *S. aureus* were observed in MUC16 knockdown cultures than in control cultures (Fig. 6). The greatest amount of *S. aureus* binding is seen on cells that by immunofluorescence show less binding of the MUC16 antibody (Fig. 6). The amount of adherent bacteria were quantified (Fig. 6E), revealing 3.6-fold higher numbers of *S. aureus* binding in the cells expressing siRNA sequence 2 ($n = 6$; $P < 0.01$). These results suggest that MUC16 protects the epithelial surface from *S. aureus* binding.

DISCUSSION

In this report, we show that MUC16 functions by forming a protective barrier to the epithelial cell surface. When MUC16 expression is suppressed in HCLE cells, loss of surface protection is observed, as shown by dye penetrance and adherence of *S. aureus*. We also present data that indicate MUC16 binds to the ERM family of proteins through a polybasic sequence of amino acids in its cytoplasmic tail, linking it to the actin cytoskeleton in the cytoplasm of microplicae membrane folds.

MUC16 is expressed on the tips of the microplicae of the ocular surface and its cytoplasmic tail has the potential to bind the actin cytoskeleton through ERMs. Therefore, we hypothesized that its presence may be necessary for the formation and maintenance of these specialized membrane structures. Our hypothesis seemed likely because of the three membrane-associated mucins expressed by the ocular surface, MUC16 is the only one to bind the moesin construct. However, the siRNA-mediated knockdown of MUC16 did not alter the number and length of apical membrane extensions on the surfaces of HCLE cells, indicating that the hypothesis was incorrect. Overexpression of CD43 and CD44 in fibroblasts has been shown to induce the formation of surface projections on fibroblasts through linkage to ERMs and the cytoskeleton. This effect, however, was not seen in epithelial cells,[27] suggesting

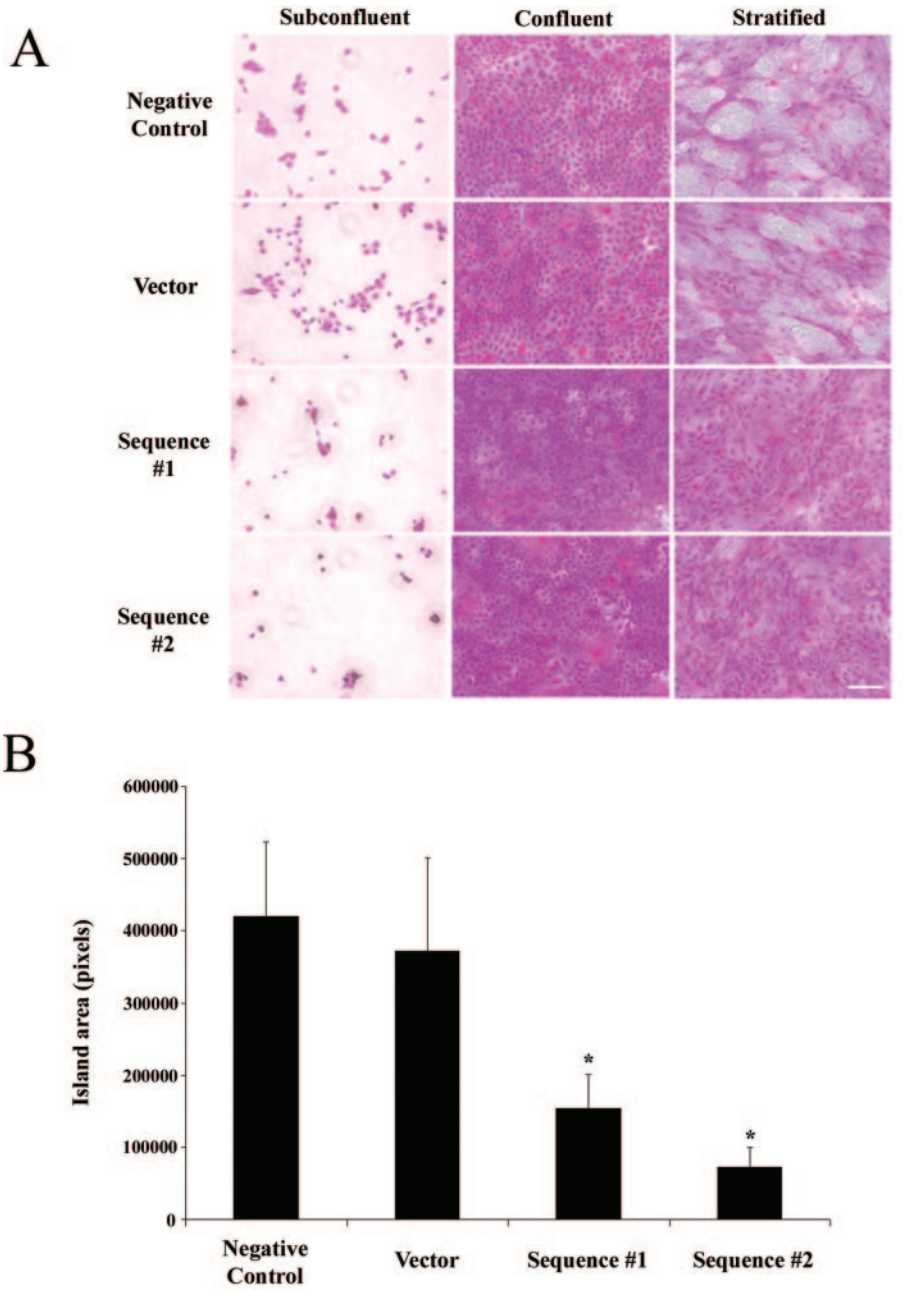

FIGURE 5. Rose bengal penetrance in HCLE cells with MUC16 knockdown. (**A**) Brightfield micrographs of transfected HCLE cells showing change in the binding pattern of the anionic vital dye rose bengal. Cells were photographed using phase-contrast microscopy. Scale bar, 50 μm. (**B**) Quantification of decrease in the amount of island area that excludes rose bengal. Areas of islands were quantified using image analysis software ($n = 5$; $^*P < 0.01$).

that modification of expression of even two single membrane-spanning molecules in epithelial cells is insufficient to alter microvillus formation and structure and that multiple membrane-tethered molecules associate with ERMs for their formation. Perhaps a critical density and diversity of membrane-associated molecules on the epithelial surface are required for the formation of microplicae and microvilli by linkage to the actin cytoskeleton. In contrast, other cytoplasmic actin-binding proteins may be required to tether the cytoskeleton to the intracellular phospholipid layer of the plasma membrane on surface projections, e.g., myosin I, which has been shown to be critical for the formation of normal microvilli structure.[50]

MUC16 is a component of the ocular surface glycocalyx and is thought to participate in providing a barrier to the epithelial surface,[45] though direct evidence has not been available. We chose to examine the effect of MUC16 on the epithelial barrier using rose bengal, a dye thought to penetrate regions of the ocular surface epithelium in which protection has been compromised,[44] perhaps by alterations in shedding, expression, or glycosylation of membrane-associated mucins. The dye is used clinically as an assessment of damage to the ocular surface epithelium and as an indication of dry eye. Indirect evidence attributes protection from rose bengal penetration to the capacity of stratified islands to produce MUC16 and the T-antigen O-carbohydrate, thus shielding the ocular surface epithelia.[45] The fact that knockdown of MUC16 from stratified HCLE cells reduces this protection, as indicated by dye penetrance, definitively supports the hypothesis that MUC16 helps form a protective barrier to the epithelial surface.

To further elucidate the effect of MUC16 in providing a barrier to the apical epithelial surface, we show that MUC16 provides protection to HCLE cells from pathogen adherence (*S. aureus*). These results correlate with the hypothesis that membrane-associated mucins provide a barrier to the epithelial cell surface against pathogen adhesion.[29,51] For example, MUC1 knockout mice are more susceptible to bacterial conjunctivitis,

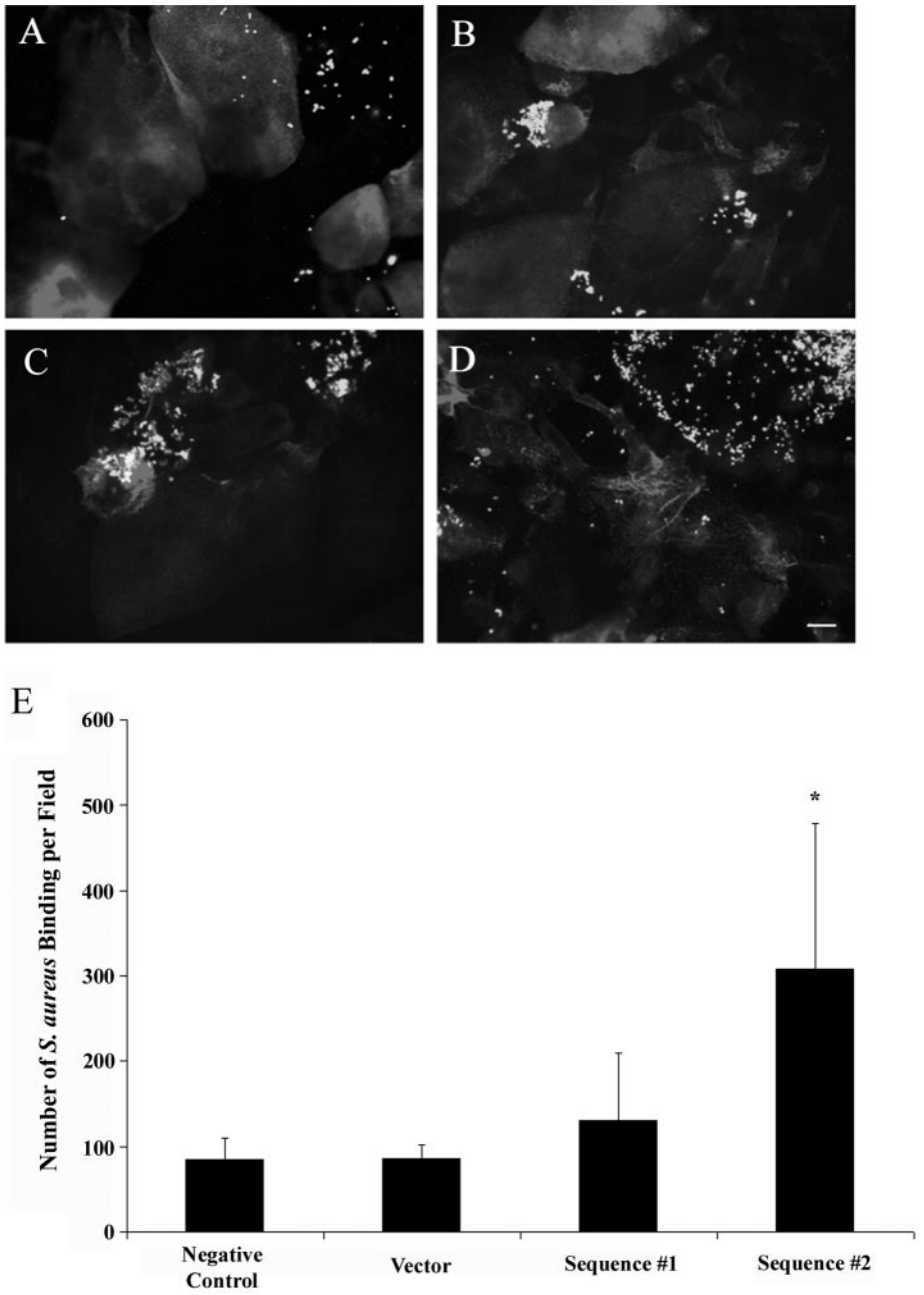

FIGURE 6. Binding of *S. aureus* to HCLE cells with MUC16 expression knockdown. (**A–D**) Immunofluorescence microscopy showing localization of MUC16 expression (*gray*) and FITC-labeled *S. aureus* (*white dots*) in nonpermeabilized HCLE cells that were (**A**) nontransfected, (**B**) vector-transfected, (**C**) MUC16 siRNA sequence 1-transfected, or (**D**) MUC16 siRNA sequence 2-transfected. Scale bar (**A–D**), 25 μm. (**E**) Quantification of *S. aureus* binding assay showing number of adherent bacteria per field ($n = 6$; $^*P < 0.01$).

presumably because of partial loss of the barrier to the epithelial surface.[29] These data are in contrast to the observation that other membrane-associated mucins, such as MUC1, appear to serve as adhesion sites for *Psuedomonas aeruginosa*.[52] This discrepancy may be explained by the fact that *Pseudomonas* has been found to interact with MUC1 through the surface molecule flagellin, which is not found in *S. aureus*.[53]

Several limitations that may affect interpretation of the results in this study include the fact that RNAi technology is incapable of completely knocking out gene expression, though the presence of MUC16 on the apical cell surface can be significantly reduced. In addition, although HCLE cells express the same membrane-associated mucins found in native ocular surface epithelia (MUC1, MUC4, MUC16), expression is not uniform throughout the culture. This is demonstrated by the fact that islands of protection, rather than complete exclusion, from rose bengal penetration are observed, as observed in intact native ocular surface epithelium.[45] These islands are groups of stratified cells that express MUC16 and mimic the terminally differentiated cells of native stratified epithelia. Although the culture model has limitations, there is sufficient similarity with the native epithelium to test our hypothesis on barrier function.

Our data present some of the first evidence for specific functions of MUC16 on the corneal epithelial cell surface. Some redundancy in barrier and disadhesive function may exist between the different membrane-associated mucins expressed on the apical cell surface because of the similarities in sequence and structure of their extracellular domains. This could be assessed through the creation of epithelial cell lines with knockdown of multiple membrane-associated mucins. However, MUC1 and MUC4 have unique functions through their cytoplasmic tail signaling capabilities and EGF-like domains, respectively.[9] Our data suggest that MUC16 may also have unique functions through its ERM-binding capability. These differences in properties suggest that the study of individual membrane-associated mucin function is important. To further

understand MUC16 function, additional characterization of the cytoplasmic tail of MUC16 is needed, including the effects of phosphorylation on intracellular signaling. These data will provide better understanding of the multifunctional role of MUC16 on the corneal epithelial surface.

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