Published in final edited form as:
Exp Eye Res. 2010 June ; 90(6): 655–663. doi:10.1016/j.exer.2010.02.014.

Membrane-tethered mucins have multiple functions on the ocular surface

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Abstract

Membrane-tethered mucins are large glycoproteins present in the glycocalyx along the apical surface of all wet-surfaced epithelia of the body, including that of the ocular surface. Originally thought to function only in epithelial surface lubrication and hydration, data now indicate that the mucins are multifunctional molecules, each having unique as well as common functions. This review summarizes current knowledge regarding the three major membrane mucins of the ocular surface, MUC1, MUC4, and MUC16. The mucins vary in their ocular surface distribution, size, structural motifs, and functions. The ectodomains of each are released into the tear film and are, thus, a component of the soluble mucins of the tear film. Both animal and in vitro models for their study are herein described, as are alterations of the mucins in ocular surface disease.

Keywords

Membrane-tethered mucins; MUC1; MUC4; MUC16; corneal epithelium; conjunctival epithelium; cell surface mucins

1. Introduction

The smooth, wet tear film over the surface of the cornea provides the major refractive power of the visual system. The wet surface with its underlying epithelium, is vulnerable to damage from foreign substances and pathogens that can enter the surface of the eye due to its direct exposure to the outside environment. The tear film lubricates the surface of the eye, protects the surface of the cornea, and is composed of water, lipids, mucins, and antimicrobial substances, which are products of the ocular surface epithelia in the Ocular Surface System (Gipson, I.K., 2007). The apical surfaces of the corneal and conjunctival epithelia act as a boundary between the epithelial layer and the tear film. The surfaces of these epithelia are comprised of numerous minute membrane folds called microplicae. Membrane-tethered mucins emanate from the apices of these microplicae to form a layer known as the glycocalyx (Gipson, I.K., 2004). A schematic representation of the structure of the tear film in relation to the membrane mucin-rich glycocalyx of the apical surface of the corneal and conjunctival epithelium is illustrated in Figure 1 (Gipson, I.K., 2004).

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Mucins are highly O-glycosylated glycoproteins present on the apical surfaces of all wet-surfaced epithelia of the body including the ocular surface, the respiratory, gastrointestinal and urogenital tracts, and the middle ear epithelium. Mucins are a family of at least 20 different glycoproteins that have two defining characteristics (for review see (Gendler, S.J. and Spicer, A.P., 1995; Hollingsworth, M.A. and Swanson, B.J., 2004)). First, they have tandem repeats of amino acids in their protein backbone that form the so-called mucin domain. The repeats are rich in serine and threonine residues that serve as sites for O-glycosylation. The second defining feature of mucins is the extensive O-glycosylation that accounts for 50–80% of the mass of the molecule (Gendler, S.J. and Spicer, A.P., 1995).

Mucins identified in humans are designated as MUCs 1, 2, 3, etc., in order of discovery, mouse homologues are identified by Muc and rat homologues by rMuc. They can be divided into two subfamilies, 1) the secreted mucins and 2) the membrane-tethered mucins. Among the secreted mucins, five are polymeric mucins, MUCs 2, 5AC, 5B, 6, and 19, the largest known glycoproteins in the body, and two are non polymeric, small, soluble mucins including MUCs 7 and 9 (for review see (Gendler, S.J. and Spicer, A.P., 1995; Moniaux, N. et al., 2001)).

The polymeric mucins possess cysteine-rich regions at both their C and N termini. These regions form intermolecular disulfide bonds that allow huge homomultimeric assemblies characteristic of mucus in the gastrointestinal and respiratory tracts. At the surface of the eye, MUC5AC is the major secreted mucin and is a product of the conjunctival goblet cells. In the tears however, secreted MUC5AC is of lower molecular weight, indicating that the mucins do not form huge multimers on the ocular surface, perhaps preventing formation of viscous mucus that could induce light scatter within the tear film (Spurr-Michaud, S. et al., 2007).

The membrane-tethered mucins, also known as cell surface, transmembrane, or membrane-spanning mucins, are characterized by a single membrane-spanning domain, a large extracellular domain, and a short cytoplasmic tail. The extracellular domain of these mucins is composed of the tandem repeat mucin domain region containing large numbers of serine and threonine residues that are O-glycosylated. This review is limited to the major membrane-tethered mucins expressed on the human ocular surface epithelia.

2. Membrane mucins of the ocular surface

Of the membrane-tethered mucins MUCs 1, 3A, 3B, 4, 11, 12, 15, 16, 17, and 20, three have been identified as major membrane-tethered mucins on the ocular surface (Figure 2). These include MUCs 1, 4, and 16, which will be the focus of this review. Message for MUCs 15 and 20 are also expressed on the ocular surface epithelium, but unlike 1, 4 and 16, their cellular origin and distribution in the epithelia are unknown. Glycogene expression microarrays have indicated that MUC20 is one of the most highly expressed glycogenes in the human conjunctival epithelium (Mantelli, F. et al., 2009).

The membrane-bound mucins are smaller in molecular weight than their large, polymeric, secreted mucin counterparts. MUC1, the smallest of the three ocular surface membrane mucins, is about 120–300 kDa; MUC4, of intermediate size, is about 900 kDa, and MUC16, the largest, is around 20 MDa (for review see (Hattrup, C. and Gendler, S., 2008)). The molecular weights of all mucins vary due to genetic polymorphisms between individuals in the number of tandem repeats in their protein backbone. Since the alleles of mucin genes are co-dominantly expressed, there can be considerable variation in sizes of the mucins within the population (for review see (Gendler, S.J. and Spicer, A.P., 1995)).

The membrane mucins that form the dense glycocalyx layer on the apical surface of the corneal and conjunctival epithelia (Gipson, I.K., 2004), due to their large size, can extend up to 500 nm from the epithelial surface (Bramwell, M.E. et al., 1986; Hilkens, J. et al., 1992). Data from

freeze-fracture studies of gut epithelium indicate that they form rod-like straight extensions away from the surface membrane (Figure IC). The extracellular domains of these membrane-tethered mucins are constituitively released into the tear film, thus their ectodomains constitute a component of mucins within tear fluid.

Post synthesis in the endoplasmic reticulum, the mucin proteins travel toward the Golgi where they undergo O-glycosylation, the characteristics of which vary with epithelial type and cell lineage. The variation is due to genetic polymorphisms and differential patterns of expression of glycosyltransferases in epithelia. O-glycosylation can occur in a linear or branched manner posttranslationally and begins with addition of N-acetylgalactosamine to serine or threonine residues, leading to the formation of GalNac residues. The N-acetylgalactosamine transferases that catalyze the initial step of glycosylation also dictate the position of O-glycosylation in the protein structure (Wandall, H.H. et al., 1997). Following elongation, termination of glycosylation occurs by addition of fucose, galactose, GalNac or sialic acid residues to the mucin protein.

Recent characterization of the membrane-tethered mucins indicates that they are multifunctional molecules (for review see (Hollingsworth, M.A. and Swanson, B.J., 2004)). They are believed to be involved in barrier function, protection and lubrication of the ocular surface (Gipson, I.K., 2004), signaling, and, hypothetically, are osmosensors (de Nadal, E. et al., 2007). Characterization of functions of individual membrane mucins is currently an active area of research in the field.

3. MUC1

Of the membrane-tethered mucins, MUC1 has been the most studied, not only because it was the first mucin to be cloned and analyzed, but also because it is a breast tumor cell marker. Among the membrane mucins, it is the most ubiquitously expressed. It is expressed by both corneal and conjunctival epithelia (Inatomi, T. et al., 1995), as well as by lacrimal gland (Paulsen, F. et al., 2004), and lacrimal duct epithelia (Paulsen, F.P. et al., 2003). Curiously, in the cornea and conjunctiva, message for the mucins is present in all cell layers of the epithelium; however, the protein is localized to the apical surface of the epithelium (Inatomi, T. et al., 1995).

3.1. Structure of MUC1 (See Figure 2)

The tandem repeat region of MUC1 contains 20–125 repeats of a 20 amino acid sequence (Lan, M.S. et al., 1990). Between the tandem repeat domain and the transmembrane domain of the molecule, one SEA domain, a Sea urchin sperm protein Enterokinase and Agrin module, is present. SEA modules are present in the extracellular domains of many membrane-spanning molecules in which ectodomain release occurs and are capable of self cleavage (Bork, P. and Patthy, L., 1995; Levitin, F. et al., 2005; Wreschner, D.H. et al., 2002). In the endoplasmic reticulum, immediately post synthesis, MUC1 undergoes proteolytic cleavage between the extracellular domain and the transmembrane domain at the FRPG/SVVV site of the SEA module (Parry, S. et al., 2001). Post cleavage, the two regions of MUC1 reassociate by non-covalent interaction.

The cytoplasmic tail of MUC1 has 74 amino acids including a number of amino acids that can be phosphorylated. Three amino acids, Cys-Gln-Cys (CQC), of the cytoplasmic tail adjacent to the transmembrane domain are known to play an important role in the targeting of MUC1 to the plasma membrane of the cell (Kinlough, C.L. et al., 2006). The CQC sequence undergoes palmitoylation and mutation of the sequence to AQA prevents MUC1 recycling (Kinlough, C.L. et al., 2006).

Apart from the full-length MUC1, truncated forms of the protein lacking either the tandem repeat region (MUC1/X, MUC1/Y or MUC1/Z) or the cytoplasmic tail, or both, have been identified, primarily in cancer cells (for review see (Gendler, S.J., 2001)). These forms could be generated by alternative mRNA splicing of MUC1; these splice variants have been identified in ocular surface epithelia (Imbert, Y. et al., 2006).

3.2. Functions of MUC1

Almost all that is known of MUC1 function is a result of study of breast cancer cells in vitro, and there is very little direct information on its function at the ocular surface. As reported in in vitro studies, three functions have been ascribed to MUC1—data indicate that it is an anti-adhesive molecule, a signaling molecule, and that it provides a barrier to or responds to pathogens.

3.2.1. Anti-adhesion—MUC1 has been recognized as an anti-adhesive molecule. Overexpression of MUC1 is frequently observed in breast carcinoma cells, resulting in lower cell-cell and cell-extracellular matrix adherence (Hilkens, J. et al., 1995). High levels of MUC1 also lead to decreased integrin-mediated cell adhesion to extracellular components in melanoma cells, transformed epithelial cells, and normal breast epithelial cells (Wesseling, J. et al., 1995). Important components that mediate MUC1's anti-adhesive properties include the length of its extracellular domain, with its heavily glycosylated tandem repeat region, and loss of its cellular apical membrane polarization in cancer cells, which interferes with cadherin-mediated cell-cell adhesion (Hilkens, J. et al., 1995; Wesseling, J. et al., 1996).

3.2.2. Signaling—The cytoplasmic tail of MUC1 (MUC1 CT) has been associated with a number of signaling events (for review see (Singh, P.K. and Hollingsworth, M.A., 2006)). Interactions of MUC1 CT with β-catenin, catenin p120, ER-α, and p53 have been demonstrated. Catenins are mediators of cell-cell adhesion and transcription, and translocate proteins to the nucleus. MUC1 cytoplasmic tail-β-catenin complexes have been observed in breast carcinoma cells, in human pancreatic cancer cells, and in airway epithelial cells. MUC1 CT-β-catenin complexes are observed within both the cytoplasm and the nucleus in pancreatic cancer cells, indicating that the MUC1 CT is transported to the nucleus when freed from the cell membrane. The downstream effects of this association are currently under study; one report has indicated that the complex inhibits proliferation of tracheal epithelial cells. The MUC1 CT in the breast cancer cell lines can associate with the signaling molecule GrB2, which in turn interacts with SOS-1, a protein involved in signaling that controls cell growth and differentiation.

The cytoplasmic tail has numerous potential sites of phosphorylation, including serine, threonine and tyrosine residues, and studies indicate that some residues are indeed phosphorylated (Gendler, S.J., 2001; Pemberton, L. et al., 1992). Tyrosine phosphorylation in the MUC1 CT has been observed in breast (Zrihan-Licht, S. et al., 1994) and ovarian cancer cell lines, which correlates to changes in cell-cell adhesion (Quin, R.J. and McGuckin, M.A., 2000). Tyrosine phosphorylation of MUC1 CT appears to be essential for the downstream activation of the ERK1/2 pathway in airway epithelial cells (Wang, H. et al., 2003). Recently a study indicated that MUC1 tyrosine residues can be phosphorylated by Met, a receptor tyrosine kinase in cancer cells that leads to increased interactions of MUC1 CT with p53 and, ultimately, a decrease in transcription of MMP-1 (Singh, P.K. et al., 2008). Serine and tyrosine residues on the MUC1 CT are constituitively phosphorylated in CHO cells (Lillehoj, E.P. et al., 2004), and serine phosphorylation is observed in the cytoplasmic tail of the MUC1/Y isoform (Baruch, A. et al., 1999). The mechanistic aspects of MUC1 CT tail phosphorylation are currently being evaluated in several models to better understand downstream signaling events and the cellular processes that the signaling affects.

3.2.3. Pathogen barrier function—Membrane-tethered mucins have been hypothesized to play an active role in pathogen barrier function. The role of MUC1 in pathogen adherence studied with respect to the pathogen *Pseudomonas aeruginosa* has, however, yielded ambiguous results. MUC1 on the cell surface has been shown to serve as a binding site for Pseudomonas aeruginosa flagellin in Chinese hamster ovary cells that were transfected with full-length MUC1 (Lillehoj, E.P. et al., 2001; Lillehoj, E.P. et al., 2002). Phosphorylation of the MUC1 CT is stimulated by the binding of *Pseudomonas aeruginosa* to the cell surface, suggesting that MUC1 is "sensing" pathogen adherence (Lillehoj, E.P. et al., 2004). It has been hypothesized that MUC1 binds the pathogen and, through ectodomain release, clears the pathogen from the cell surface. However, in vivo data on Muc1 knockout mice show lower adherence of the bacterium and increased bacterial clearance of pulmonary airways (Lu, W. et al., 2006). Inflammatory mediator expression through the signaling of *Pseudomonas aeruginosa* to the Toll-like receptor family is suppressed by increased expression of Muc1 (Lu, W. et al., 2006; Ueno, K. et al., 2008). Thus expression of MUC1 may suppress inflammatory response to bacterial infections.

As regards the ocular surface in Muc1 null mice, the eyes appear normal (Danjo, Y. et al., 2000). Of two conflicting studies, one reports increased ocular surface infection in $Muc1^{-/-}$ mice (Kardon, R. et al., 1999), while the other reports no increase (Danjo, Y. et al., 2000). The animals with increased infection were reared in an animal facility that was less "clean" than that of the second study, which may explain the conflicting data.

4. MUC4

Characterization of a membrane-tethered mucin, now known as rMuc4, that was originally isolated from rat ascites tumor cells led to the characterization of human MUC4. Two subunits of the mucin were described in rat ascites fluid and originally termed ascites sialoglycoprotein (ASGP) -1 and ASGP -2, (for review see (Carraway, K.L. et al., 2002)). Subsequent cloning of human MUC4 demonstrated that ASGP-1 and -2 are the rat homologue of the mucin. The ASGP-2 subunit and the human MUC4-beta subunit have a 70% sequence homology (Nollet, S. et al., 1998), whereas, there is a 62% similarity between ASGP-1 and MUC4 at the nucleotide level, and 59% similarity at the protein level (Nollet, S. et al., 1998). Much of the published information on the structure and function of the mucin is based on the rat homologue rMuc4, although recently several labs have focused on MUC4, particularly as it relates to human carcinomas.

The mucin is widely expressed, being present on the ocular surface as well as apical epithelial surfaces of the respiratory tract, specific regions of the gastrointestinal tract, and both female and male reproductive tracts. At the ocular surface, MUC4 message and protein is predominant in the conjunctival epithelium, with a diminution in amount of the mucin in the peripheral corneal epithelium, and little if any in central corneal epithelium (Inatomi, T. et al., 1996). By comparison in the rat, rMuc4 mRNA is expressed abundantly by both the corneal and conjunctival epithelia (Tei, M. et al., 1999) and similarly ASGP-2 protein is present all across the conjunctival and corneal epithelia (Price-Schiavi, S.A. et al., 1998).

4.1. Structure of MUC4

MUC4 is similar to MUC1 in that it is cleaved in the endoplasmic reticulum forming MUC4-α and MUC4-β, which reassociate to form a non-covalently linked heterodimer. MUC4 ectodomain contains a tandem repeat region of 145–395 repeats of 16 amino acids (Figure 2), and an N-terminal region comprised of a sequence of imperfect repeats. The ectodomain also contains a cysteine-rich site, a nidogen homology sequence, and a von Willebrand factor type D sequence close to the transmembrane domain (Figure 2) (for review see (Hattrup, C. and Gendler, S., 2008)). The proteolytic cleavage site of MUC4 that yields the two subunits MUC4-

α and MUC4-β is predicted to be at Gly-Asp-Pro-His and is present adjacent to the cysteine-rich regions of the molecule. The ectodomain region near the transmembrane domain of MUC4 is a region rich in N-glycosylation, which contains three EGF binding domains. A transmembrane domain and a 22 amino acid cytoplasmic tail form the C-terminus of the molecule. Potential, but currently undemonstrated, sites of phosphorylation in the MUC4 cytoplasmic tail include one tyrosine residue and three serine residues. Of the three major membrane-tethered mucins at the ocular surface, MUC4 is the only one that lacks a SEA module; however, it undergoes ectodomain release despite the lack of the module, as it is present in the tear film (Spurr-Michaud, S. et al., 2007).

4.2. Function of MUC4

As with MUC1 and other membrane mucins, MUC4 through its heavily glycosylated tandem repeat domain is assumed to provide a lubricating protective glycocalyx component at the surface of the epithelia, albeit direct evidence is lacking. A second function of the molecule, signaling through the MUC4-β EGF domains, has been studied more extensively.

4.2.1. Signaling—Studies conducted in rat indicate that rMuc4, through its EGF domains, associates with and activates the receptor tyrosine kinase ErbB2, a molecule that induces epithelial cell proliferation post epithelial damage (for review see (Carraway, K.L. et al., 2002)). The interaction between ASGP-2 and ErbB2 (HER2) and ERB3 to induce apoptosis has been described in rat lacrimal gland tissue (Arango, M.E. et al., 2001). A recent publication has shown that receptors ErbB2 and -3 are phosphorylated in response to injury to airway epithelial cells, thus the formation of a MUC4-ErbB2 complex could be a mechanism by which cells sense epithelial damage (Theodoropoulos, G. et al., 2009). The MUC4-ErbB2/ErbB3 complex has been hypothesized to protect cancerous cells from undergoing apoptosis (Carraway, K.L. et al., 2002).

4.2.2. Other functions—On the rat ocular surface, the sialomucin complex is expressed in the corneal and conjunctival epithelium, and a soluble form of the sialomucin complex is present in the tear fluid (Price-Schiavi, S.A. et al., 1998). It has been hypothesized that rMuc4 could play a role in the maintenance of tear fluid stability (Price-Schiavi, S.A. et al., 1998). Similar to MUC1, rMUC4 is also known to act as an anti-adhesive molecule, preventing integrin-mediated cell adhesion in breast carcinoma cells (Komatsu, M. et al., 1997). rMuc4 sialomucin complex is present at high levels in desquamating corneal epithelial cells and has been hypothesized to play a role in desquamation (Lomako, J. et al., 2005). Thus at the ocular surface of rodents, rMuc4 may act as an anti-adhesive and could play a role in tear film stability; however, its function at the human corneal surface is not known.

5. MUC16

MUC16, originally isolated from ovarian tumor cells, was known as the ovarian tumor cell marker CA125 prior to its cloning and protein characterization (for review see (Perez, B.H. and Gipson, I.K., 2008)). In addition to its expression by ocular surface epithelia, MUC16 is expressed at the surfaces of the tracheal/bronchiolar epithelium, the female reproductive tract, and the mesothelium of the abdominal cavity. At the ocular surface, the mucin is expressed by apical cells of the corneal and conjunctival epithelium, as well as by the lacrimal gland ductal epithelium. Its corneal expression levels appear particularly high relative to the conjunctiva (Argueso, P. et al., 2003).

5.1. Structure of MUC16

MUC16 is a 20 – 25 MDa molecule that has 22,152 amino acids in its protein sequence (O'Brien, T.J. et al., 2002). The N-terminus of MUC16 is a heavily O-glycosylated non tandem

repeat domain, which consists of approximately 12,000 amino acids (Figure 2). The tandem repeat region that is adjacent to the N-terminus is composed of 60 repeats of 156 amino acids. MUC16 is unique among the membrane-tethered mucins, in that it has 56 identified SEA modules compared to 1 SEA module that is present in MUC1. The MUC16 SEA modules are made up of different sequence lengths of amino acids and are interspersed between and within the tandem repeat domain present in the molecule. The SEA modules themselves contain a large number of cysteine residues and have the potential to form disulfide bridges in inter- and intramolecular fashion (Maeda, T. et al., 2004). MUC16 also contains 14 leucine-rich repeats (Yin, B.W. and Lloyd, K.O., 2001) and 2 ANK repeats that are all woven into the tandem repeat domains along with the SEA modules. The above-mentioned regions together form the ectodomain of MUC16 (Figure 2). The released, large ectodomain without its membrane-spanning cytoplasmic tail domain, as assayed by SDS-PAGE, is approximately the same molecular weight as the whole mucin, thus a potential cleavage site could be located in the penultimate or ultimate SEA module close to the transmembrane region (O'Brien, T.J. et al., 2001). Adjacent to the last SEA module, a 21 amino acid transmembrane domain is followed by a 35 amino acid cytoplasmic tail. The cytoplasmic tail of MUC16 has several potential phosphorylation sites including three tyrosines and one serine residue (Fendrick, J.L. et al., 1997; O'Brien, T.J. et al., 2001). Phosphorylation of the potential sites has not been explored. Due to the relatively recent discovery of MUC16 (Yin, B.W. and Lloyd, K.O., 2001) and its large size, much of the structural and signaling aspects of this mucin are not known.

5.2. Function of MUC16

5.2.1. Association with cytoskeleton and potential signaling—A polybasic sequence (RRRKK) in the cytoplasmic tail of MUC16 adjacent to the transmembrane domain can bind to the actin cytoskeleton through the ezrin/radixin/moesin (ERM) family of proteins (Blalock, T.D. et al., 2007), a family of linker proteins shown to be involved in the formation of surface membrane protrusions such as microvilli. Moesin was shown to bind to a synthetic peptide mimicking the cytoplasmic tail of MUC16 but not to peptides mimicking the cytoplasmic tails of MUCs 1 or 4. Moreover, immunoelectron microscopy performed with fixed sections of human cornea indicated that MUC16 was localized on the apical surface of the microplicae and the ERMs on the cytoplasmic face (Blalock, T.D. et al., 2007), supporting the hypothesis that the ERM family interacts with the MUC16 cytoplasmic tail. Since MUC16 is localized to the tips of the membrane protrusions or microplicae on the surfaces of the corneal and conjunctival epithelia, and since its cytoplasmic tail binds to the actin cytoskeleton through the ERMs, it has been hypothesized that MUC16 may be important in microplicae formation (Gipson, I.K., 2004).

5.2.2. Barrier function in the glycocalyx—The role of MUC16 as a component of the glycocalyx barrier at the ocular surface has been explored using two methodologies. Rose bengal, an anionic dye used in dry eye diagnosis, was hypothesized to penetrate apical cells of the corneal epithelium due to injury or changes in surface mucin content. Corneal epithelial cells cultured to confluence and induced to stratify and express membrane mucins MUCs 1, 4 and 16 on their apical surface (Gipson, I.K. et al., 2003) have been shown to develop islands within the culture that prevent rose bengal dye penetrance (Argueso, P. et al., 2006). To determine the role of MUC16 in the barrier, the mucin was stably knocked down in human corneal epithelial cells using siRNA techniques followed by rose bengal staining (Figure 3). A statistical increase in the penetration of the dye into the human epithelial cells occurred with MUC16 knockdown compared to controls directly demonstrating the role of the molecule in barrier function (Blalock, T.D. et al., 2007).

One of the important functions of the epithelial glycocalyx barrier is preventing pathogen invasion into epithelia. When control HCLE cells and MUC16 knockdown human corneal

limbal epithelial (HCLE) cells were tested for adherence of *Staphylococcus aureus*, the number of bacteria that adhered to MUC16 knockdown cells was much higher than to control cells. Thus MUC16 has an important role in preventing bacterial adherence (Blalock, T.D. et al., 2007).

The mucin barrier has also been evaluated with respect to the role of O-glycan carbohydrates on the mucin protein, and the role of O-glycan binding to lectins such as galectin-3, expressed on the apical surfaces of corneal and conjunctival epithelia. Inhibition of O-glycan synthesis in epithelial cells removes the rose bengal dye penetrance barrier, demonstrating the role of O-glycans in barrier function (Argueso, P. et al., 2006). Cell surface mucins MUC1 and MUC16 bind to galectin-3 in vitro, and rose bengal dye penetrance in HCLE cells decreases in the absence of galectin-3 (Argueso, P. et al., 2009). Thus galectin-3-mucin O-glycan interactions could play a major role in formation of the mucosal barrier.

6. Ectodomain release

An interesting aspect of membrane-tethered mucins is the release of the ectodomain of these molecules from the epithelial surface as soluble forms of the mucins. Soluble forms of MUCs 1, 4 and 16 have been detected in normal human tears (Spurr-Michaud, S. et al., 2007). Mechanisms that induce this release have most often been studied in vitro.

Induced shedding of MUC1 has been explored in human uterine epithelial cells (HES) and in human corneal epithelial cells (HCLE). Agents such as phorbol 12 myristate 13-acetate (PMA) (Thathiah, A. et al., 2003) and pervanadate (Thathiah, A. and Carson, D.D., 2004) are known to induce MUC1 shedding in cells. It has been hypothesized that proteases play a role in ectodomain release, and the hypothesis has been confirmed in the case of MUC1 in HES uterine epithelial cells. PMA-stimulated MUC1 ectodomain release in these cells is mediated by a member of the matrix metalloproteinase family, TNF-α converting enzyme/a disintegrin and metalloproteinase (TACE/ADAM-17) (Thathiah, A. et al., 2003). This result has been confirmed by several experiments using the endogenous inhibitors of MMPs among which only TIMP-3 exhibited a decrease in MUC1 constituitive shedding. TIMP-3 is known to specifically inhibit TACE/ADAM-17. In vivo proof of the role of the metalloproteinase ADAM-17 in MUC1 ectodomain release was provided using TACE/ADAM deficient mice that did not constituitively shed MUC1 when stimulated with PMA (Thathiah, A. et al., 2003). Thus several lines of research indicate that TACE plays a role in constituitive MUC1 ectodomain shedding in uterine epithelial cells.

Soluble forms of the rMuc4/sialomucin complex are detected in the saliva and the tear fluid (Arango, M.E. et al., 2001). The ectodomain of rMuc4/SMC is released into the culture medium of normal epithelial cells, breast cancer cells, melanoma and kidney cells by a “proteolytic cleavage mechanism” of the full-length rMuc4 (Komatsu, M. et al., 2002). Human MUC4 has an amino acid sequence that is 70% homologous to rMuc4 (ASGP-2), thus experiments on HBL-100 epithelial cells and COS-7 cells corroborated the results observed in the rMuc4 model, indicating a “proteolytic cleavage mechanism” for the generation of soluble MUC4 (Komatsu, M. et al., 2002). Intracellularly, “proteolytic cleavage” of rMuc4 has been found to occur between the two EGF-like domains of the molecule early in the biosynthesis pathway, which correlates to the cleavage of rMuc4 into the ASGP-1 and -2 subunits, but it is not clear if the ectodomain release site is the same as that of intracellular cleavage (Komatsu, M. et al., 2002).

MUC16 has been observed in human tears and in culture media of human corneal and conjunctival epithelia. Blalock et al. (2008) have shown that the MUC16 in HCLE culture media and in tears lacks the cytoplasmic tail and is, thus, a product of ectodomain release or shedding (Blalock, T.D. et al., 2008). Inflammatory mediators including neutrophil elastase,

matrix metalloproteinases, MMP-7 and -9, and TNF-α (after 24-hour exposure) can induce MUC16 ectodomain release (Blalock, T.D. et al., 2008). However, this study reported that neither TACE, which mediates MUC1 shedding in uterine epithelial cells, nor PMA stimulation induced an increase in MUC16 ectodomain release in the corneal cells (Blalock, T.D. et al., 2008). Thus unlike MUC1 shedding, MUC16 shedding does appear to be mediated by a membrane-bound metalloproteinase.

7. Alterations of membrane mucins in ocular surface disease

On the normal epithelial surface, membrane mucins emanate from the apices of the microplicae and apical cell membrane, forming the glycocalyx barrier. Alterations in amounts of protein, RNA and ectodomain release of membrane-tethered mucins could change the dynamics of the barrier. Dry eye is associated with changes in mucin expression and glycosylation, and studies have shown that the mRNA levels of MUC5AC and MUC1 are decreased in patients with Sjögren's syndrome compared to normal controls (Argueso, P. et al., 2002). Distribution of the carbohydrate epitope of MUC16 is altered in patients with non-Sjögren's syndrome compared to controls (Danjo, Y. et al., 1998). This alteration could be the result of changes in glycosylation patterns of mucin ectodomain release or expression of the mucin in dry eye.

The mechanisms of alterations of surface mucins in patients with dry eye may be due to inflammatory mediators present in the tears that influence membrane mucin expression or ectodomain release. In experiments on cultured human corneal epithelium, IL-6 has been shown to downregulate levels of MUC1 protein, and IFN-γ and TNF-α induce significant increase in the levels of mRNA, cellular protein and ectodomain shedding of MUC1 (Albertsmeyer, A. et al., (In press)). Similarly TNF-α plus IFN-γ induced an upregulation of MUC16 expression, protein synthesis, and ectodomain release. Studies conducted using a spontaneously transformed conjunctival cell line have indicated an upregulation of MUC16 mRNA when exposed to cytokines IL-1α, IL-1β and INF-γ; however, the same cytokines induced a downregulation of MUC16 mRNA in primary cultures of corneal epithelium (Paulsen, F. et al., 2008). These studies, taken together, indicate the possibility for a role of inflammatory mediators in mucin expression and release at the ocular surface and, therefore, may be responsible for the alterations in membrane mucins seen in the dry eye diseases.

8. Models for the study of membrane mucins of the ocular surface

8.1. Rodent models for study of mucins

Rodent models have been used to investigate mucin expression in induced ocular diseases, including keratinization due to vitamin A deficiency and dry eye. The effects of depletion of vitamin A on ocular surface mucin expression were studied in rats fed on a vitamin A deficient diet. The epithelium exhibited loss of rMuc4 mRNA but not rMuc1 following long-term vitamin A deficiency indicating that vitamin A was important in rMuc4 expression on the ocular surface and in preventing keratinization (Tei, M. et al., 2000). These results were corroborated in an in vitro culture model of HCjE cells treated with retinoic acid followed by analysis of the levels of membrane-tethered mucin mRNA. No changes were observed in rMuc1 mRNA, while there was a significant increase in both mRNA and protein expression of MUC4 and MUC16 with retinoic acid treatment (Hori, Y. et al., 2005). These data indicate that MUC1 is not regulated by retinoic acid, and since the mucin is not altered in this keratinizing disease, it may not be important in maintenance of the wet-surface epithelial phenotype.

Since dry eye is common to post menopausal women, the effect of estrogen and progesterone on mucin expression was evaluated in ovariectomized mice, which were either supplemented or not with estrogen, progesterone, or a combination of the hormones. mRNA of the mucins

Muc5AC and Muc4 were assayed, and analysis indicated that estrogen and progesterone do not regulate these mucins in the ocular surface epithelium of mice (Lange, C. et al., 2003).

With relevance to the levels of MUC16, an important component of the glycocalyx in human cornea, a recent study indicated that MUC16 is not present on the ocular surface of mice (Cheon, D.J. et al., 2009). Thus rodent models, although useful in the study of certain diseases, do not entirely duplicate the mucin expression profiles in humans and should be used with caution.

8.2 In vitro models for study of mucins

Since the patterns of expression of membrane mucins on the human ocular surface are different from those in rodent models, in vitro models using cell lines derived from human cornea or conjunctiva have been used to study membrane mucin function and the regulation of their expression.

The cell lines exhibit membrane mucin gene expression profiles similar to those observed in their native epithelium when cultured to induce stratification and differentiation (Gipson, I.K. et al., 2003). These cell lines, which produce MUCs 1, 4 and 16 (Gipson, I.K. et al., 2003), have been used to study functions of MUC16, ectodomain release of MUC1, -4, and -16, as well as their regulation (Albertsmeyer, A. et al., (In press); Blalock, T.D. et al., 2008; Blalock, T.D. et al., 2007). Other cell lines reported in the literature for study of mucins include an SV40-immortalized human corneal epithelial cell line that differentiates but does not stratify to the same degree as native epithelia (Araki-Sasaki, K. et al., 1995), and a spontaneously immortalized conjunctival cell line, described by Diebold et al., that does not express MUC5AC message (Diebold, Y. et al., 2003).

9. Summary and future directions

Membrane-tethered mucins of the ocular surface glycocalyx are multifunctional molecules that 1) act as a selective barrier to penetrance of molecules, 2) act as an anti-adhesive that prevents cell and pathogen adherence, 3) are believed to help lubricate and maintain tears on the ocular surface, and 4) signal through either their cytoplasmic tails or their EGF domains. The ectodomains of the three mucins are constituitively released from the apical surfaces of corneal and conjunctival epithelia, thus they contribute to the mucin component of the tear film. There is, however, little information on the mechanism of ectodomain release—an area ripe for investigation.

Information regarding specific functions of individual membrane mucins on the ocular surface epithelia is just emerging with the demonstration of MUC16's role in preventing rose bengal penetrance and pathogen adherence. Future studies investigating specific functions of MUC1 and MUC4 are needed, as are studies determining signaling capacities of all three mucins at the ocular surface.

Recently, the ectodomains of the mucins that appear in the glycocalyx barrier, have been shown to bind to galectin-3, and the galectin participates in the rose bengal dye penetrance barrier (Argueso, P. et al., 2009). Perhaps there are additional binding partners of the mucins that participate in the glycocalyx barrier.

Although it is now apparent that MUC16 helps to prevent pathogen adherence, it is also clear that pathogens can pass through the mucin barrier. It has been estimated that 80% of all infections occur across wet mucosal surfaces, such as that of the ocular surface. Understanding how pathogens can manipulate the membrane mucin barrier to gain entrance to epithelia may yield new methods to prevent infection and is an understudied area.

Finally, drying cicatrizing diseases of the ocular surface abrogate mucin production at the ocular surface. Development of methods to induce mucin expression may yield treatments for these prevalent diseases.

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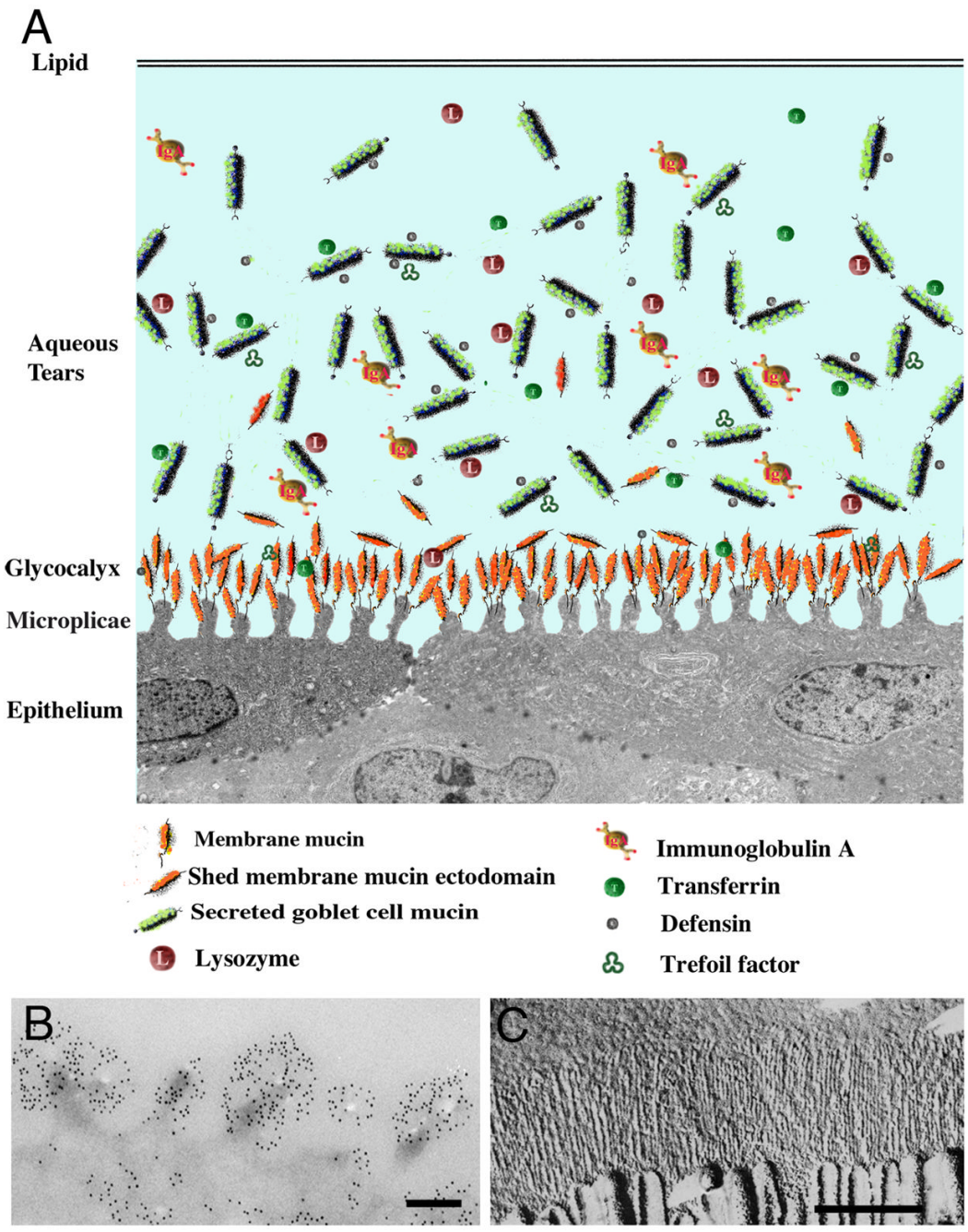

Fig. 1.

Diagram and electron micrograph images of the tear film-glycocalyx interface. (A) Diagram of the tear film demonstrating tear components and the apical surface glycocalyx with its membrane-tethered mucins (after (Gipson, I.K., 2004)). (B, C) Transmission electron micrograph showing immunogold localization of MUC16 on microplicae. Bar = 0.25 μm. (C) Freeze-fracture image demonstrating long filamentous structure within the glycocalyx of the gut epithelium that appear, based on length, to be membrane-tethered mucins (from (Swift, J.G. and Mukherjee, T.M., 1976)). Bar = 0.5 μm.

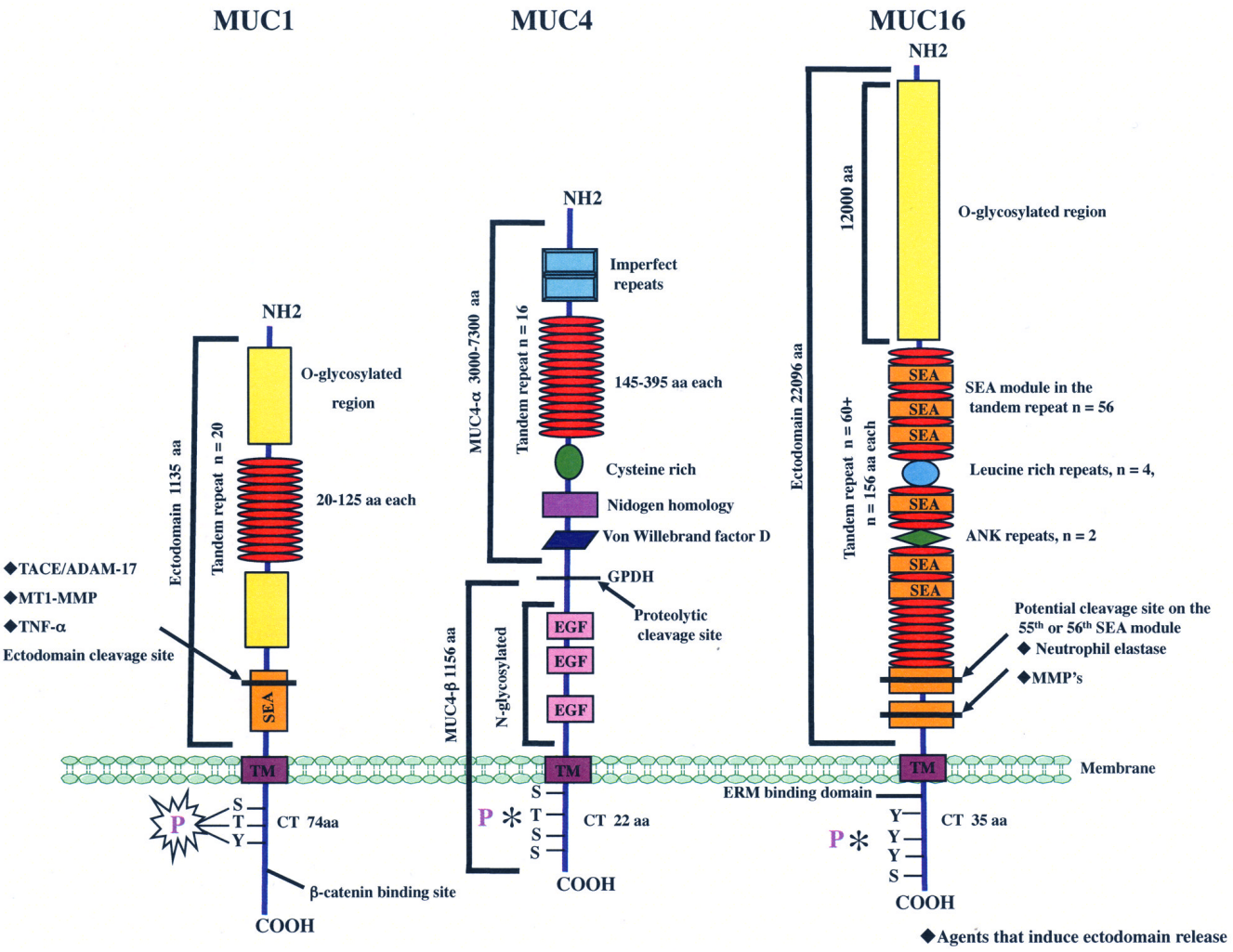

Fig. 2.

Diagram of the molecular domains of the major ocular surface, membrane-tethered mucins MUC1, MUC4 and MUC16. The membrane mucins represented share a heavily O-glycosylated ectodomain, comprised of a variable number of tandem repeats rich in serine, threonine, and proline. The amino terminal domains of MUC1 and MUC16 have a non tandem repeat, whereas the tandem repeat domain of MUC4 extends to the NH_2 terminus. MUCs 1 and 16 have 1 and 56 SEA modules, respectively, while MUC4 has none. MUC4 is the only membrane-tethered mucin that contains EGF domains. The ectodomain release sites of each mucin are indicated by an arrow, and agents that have been demonstrated to induce ectodomain release are indicated by ◆. The cytoplasmic tails (CT) of the three membrane mucins differ in length and binding partners. The cytoplasmic tail of MUC1 can be phosphorylated and contains a β-catenin binding site. The potential phosphorylation sites on MUCs 4 and 16 are indicated by *. MUC16 CT, along with potential phosphorylation, sites has an ezrin/radixin/moesin binding domain.

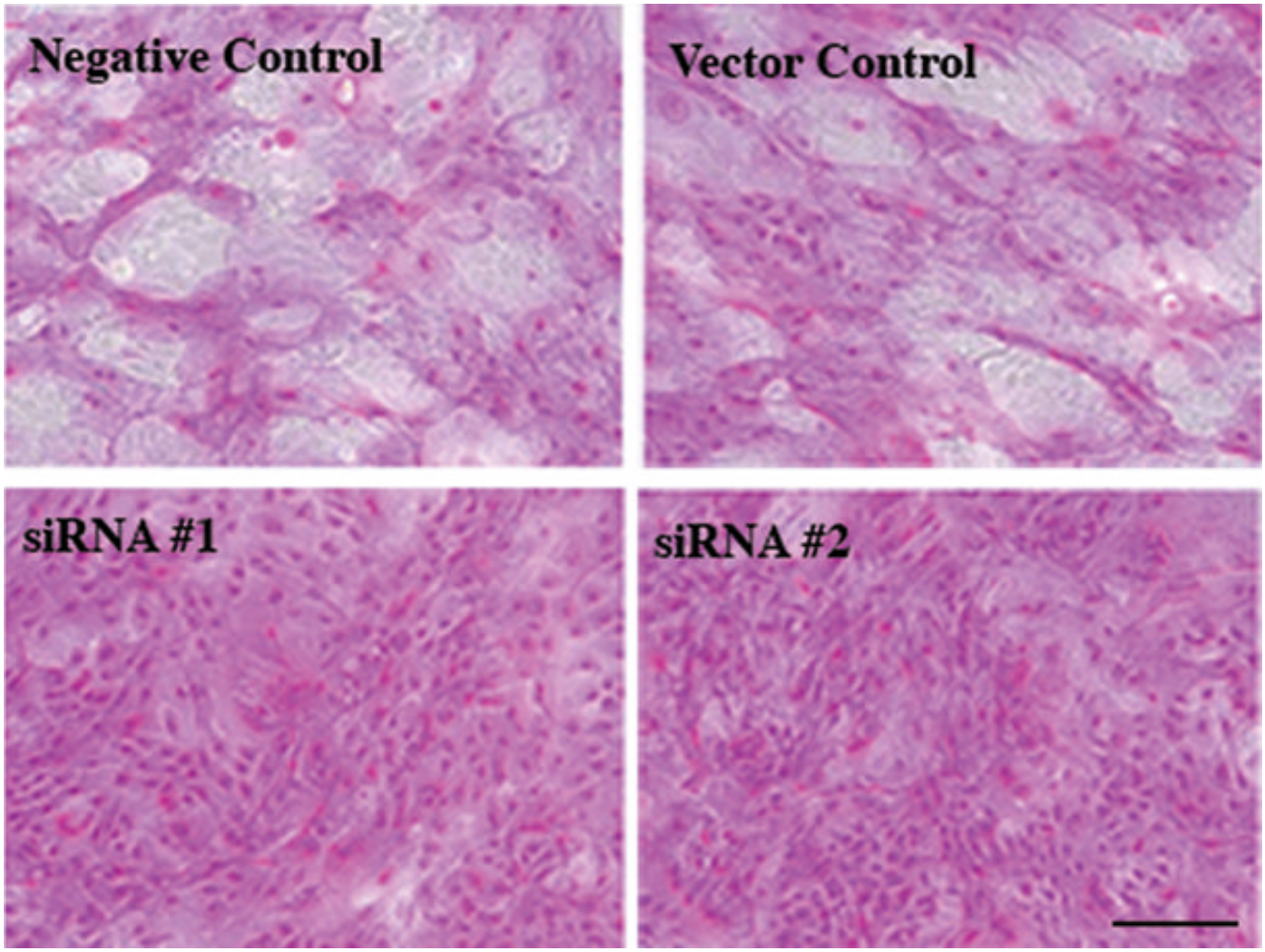

Fig. 3.

Rose bengal dye penetrance in cultured stratified HCLE cells with or without knockdown of MUC16 using siRNA methods. The untreated control and vector only control show island of cells that exclude rose bengal dye, whereas cells with MUC16 knockdown, either siRNA sequence 1 or 2, do not exclude the dye. These data demonstrate the role of the membrane-tethered mucin MUC16 in barrier function. Original data in Blalock et al., 2007 (Blalock, T.D. et al., 2007). Bar = 50 um.