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Membrane-Associated Mucins of the Ocular Surface:

New Genes, New Protein Functions and New Biological Roles in Human and Mouse

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Declaration of Interest

MEF and SJ are named as inventors on an issued United States patent entitled “Structure/Function of Clusterin Pharmaceuticals” and a pending patent application entitled “Method to Protect and Seal the Ocular Surface” (United States application 16/103,741, filed Aug 14, 2018), assigned to the University of Southern California and related to work mentioned herein. MEF is a co-founder and serves as Chief Scientific Officer for Proteris Biotech, Inc., a company focused on developing pharmaceuticals for treating eye disease. The other authors have no commercial or proprietary interest in any concept or product described in this article.

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Abstract

The mucosal glycocalyx of the ocular surface constitutes the point of interaction between the tear film and the apical epithelial cells. Membrane-associated mucins (MAMs) are the defining molecules of the glycocalyx in all mucosal epithelia. Long recognized for their biophysical properties of hydration, lubrication, anti-adhesion and repulsion, MAMs maintain the wet ocular surface, lubricate the blink, stabilize the tear film and create a physical barrier to the outside world. However, it is increasingly appreciated that MAMs also function as cell surface receptors that transduce information from the outside to the inside of the cell. A number of excellent review articles have provided perspective on the field as it has progressed since 1987, when molecular cloning of the first MAM was reported. The current article provides an update for the ocular surface, placing it into the broad context of findings made in other organ systems, and including new genes, new protein functions and new biological roles. We discuss the epithelial tissue-equivalent with mucosal differentiation, the key model system making these advances possible. In addition, we make the first systematic comparison of MAMs in human and mouse, establishing the basis for using knockout mice for investigations with the complexity of an in vivo system. Lastly, we discuss findings from human genetics/genomics, which are providing clues to new MAM roles previously unimagined. Taken together, this information allows us to generate hypotheses for the next stage of investigation to expand our knowledge of MAM function in intracellular signaling and roles unique to the ocular surface.

Keywords

Ocular surface; glycocalyx; membrane-associated mucin; signal transduction; epithelial tissue-equivalent; knockout mouse

I. Introduction

The wet ocular surface comprises the stratified squamous mucosal epithelia of the cornea/conjunctiva and the overlying tear film (Gipson, 2007). These cells are continually renewed in a process whereby daughter cells generated by division of basal cells at the basement membrane are displaced upward in the cell layers, become increasingly flattened, and undergo terminal differentiation. At the ocular surface, tight junctions form to seal the space between adjacent apical cells, creating a paracellular barrier to entry of noxious substances from the environment. In addition, the plasma membranes of apical cells develop folds called microplacae, that project outward into the tear film, and from which the mucosal glycocalyx is elaborated.

The mucosal glycocalyx constitutes the point of interaction between the tear film and the apical cells of the ocular surface epithelia. It forms a transcellular barrier to the outside world, defending and protecting, while also allowing selective penetration. Its water-holding properties maintain the wet surface and it lubricates the blink. Its interaction with the precocular tear film is stabilizing and facilitates spreading, thus, maintaining a smooth and refractive surface of high optical quality.

The word “mucin” is usually taken to mean an extracellular secretion of goblet cells, or other secretory gland cells, that forms a gel, with the primary role to coat, lubricate, and protect the epithelial surfaces of the body. However, the epithelial membrane-associated mucins (MAMs¹), expressed by most glandular and ductal epithelial cells, form a distinct mucin subgroup. MAMs serve as the major component molecules of the mucosal glycocalyx of the ocular surface and other tissues, and are the focus of this article. Both secreted and membrane-associated subgroups are encoded by members of the *MUC* gene family.

MUC family mucins are characterized by a series of tandem amino acid repeats of identical or highly similar sequence, rich in serine, threonine and proline residues. The serine and threonine residues serve as the site for O-linked glycosylation, and O-linked oligosaccharide chains account for 50–90% of the mass of the molecule. Tandem repeats are also found in other mucins, but the sites for O-linked glycosylation are not as densely spaced, meaning that glycans comprises less of the total mass. Biophysical properties of mucins are largely determined by the extent and nature of the O-linked glycosylation rather than to the polypeptide sequence itself (with the exception of the serines/threonines that are modified) (Argueso and Gipson, 2001). The multiplicity of the mucin tandem amino acid repeats amplifies the properties of mucins dependent on these structures, in both secreted mucins and MAMs (Hollingsworth and Swanson, 2004).

Much of the early interest in MAMs was driven by their pathological roles in cancers. Attempts to develop antibodies recognizing tumor-associated antigens in the 1980s led to identification of high molecular weight glycoproteins with the properties of mucins. Biochemical preparations proved to be heterogenous in composition, thus, determination of mucin amino acid sequences purified by conventional biochemical purification methods of the time proved difficult. The development of antibodies reactive with specific core protein epitopes provided the means for purification of individual mucins by affinity chromatography, enabling cloning of their cDNAs and genes. This provided the probes needed for study of mucin roles in cancer, as well as other mucosal tissues.

Molecular cloning of the first *MUC* gene was reported in 1987 (Gendler et al., 1987). In 1990, an international workshop was held in San Francisco, California with the goal to sort out the numerous antibodies that had been generated (Taylor-Papadimitriou, 1991). At about that time, the naming convention for the *MUC* gene family became established. In the 2007 Friedenwald Award Lecture, Dr. Ilene Gipson describes the process of characterizing MAMs of the ocular surface mucosal glycocalyx, first using a monoclonal antibody developed in her lab, then using probes from other labs, as they became available (Gipson, 2007).

A number of review articles have provided perspective on the field over the years, with regard to cancers and various organ systems (Apostolopoulos and McKenzie, 1994; Apostolopoulos and McKenzie, 2017; Bafna et al., 2010; Bhavanandan, 1991; Carraway et

¹Abbreviations: HUGO nomenclature used for genes and their products. CT: cytoplasmic tail; ED: extracellular domain GRC: Genome Reference Consortium; HGNC: HUGO Gene Nomenclature Committee; MAM: membrane-associated mucin; MPS: multipurpose contact lens cleaning solution; NCBI: National Center for Biotechnology Information; RT-PCR: reverse transcription-polymerase chain reaction; SEM: scanning electron microscopy; TEM: transmission electron microscopy; VNTR: variable number tandem repeats

al., 2007; Carraway et al., 2003; Gendler and Spicer, 1995; Gendler et al., 1991; Gum, 1992; Hattrup and Gendler, 2008; Hilkens et al., 1992; Hollingsworth and Swanson, 2004; Kim, 2012; Moniaux et al., 2001; Rose, 1992; Seregini et al., 1997; Singh and Hollingsworth, 2006; Strous and Dekker, 1992; van Putten and Strijbis, 2017; Xing et al., 2000; Xing et al., 2001). This includes the ocular surface (Ablamowicz and Nichols, 2016; Argueso, 2013; Argueso and Gipson, 2001; Baudouin et al., 2018; Gipson, 2004, 2007; Gipson and Argueso, 2003; Gipson et al., 2004; Gipson and Inatomi, 1998; Govindarajan and Gipson, 2010; Guzman-Aranguez and Argueso, 2010; Jentoft, 1990; Mantelli and Argueso, 2008; Mantelli et al., 2013).

The current article provides an update for the ocular surface, placing it into the broad context of findings made in other organ systems, and including new genes, new protein functions, and new biological roles. We discuss the epithelial tissue-equivalent with mucosal differentiation, the key model system that have made these advances possible. In addition, we make the first systematic comparison of the MAMs in human and mouse, establishing the basis for using transgenic and knockout mice for the next phase of discovery. We conclude with a section on findings from human genetics, which have generated the proposal of intriguing new MAM roles that were previously unimagined.

II. Genes, Gene Expression, Protein Localization

A. General

Table 1 lists the currently recognized twenty-one genes of the human Mucin (MUC) gene group, as defined by the HUGO Gene Nomenclature Committee (HGNC). There is no *MUC18* in this series because it is a well-published alias for the unrelated gene *MCAM*. It should be noted that the existence of a separate *MUC3A* and *MUC3B* gene in the human genome is still under investigation (personal communication, Dr. Eric Cox, National Center for Biotechnology Information (NCBI)). We continue to list both genes here (as does the HGNC website), but discuss only *MUC3A* going forward in this article.

The genes in Table 1 are ordered by chromosomal location, and are subdivided into those that encode secretory mucins (gel-forming and soluble), and those that encode membrane-associated mucins (MAMs).

Eight of the genes listed in Table 1 encode secretory mucins. The products of four of these genes form extremely large oligomeric gels through linkage of protein monomers via disulfide bonds. There are also four secretory mucins that do not form oligomeric gels. The gel-forming mucin OVGPI, is primarily expressed by oviduct epithelial cells. The others are expressed by many different mucous epithelia. Protein products of *MUC5AC*, *MUC2*, *MUC5B* and *MUC7* have been detected in the ocular surface epithelia and/or in the lacrimal gland (reviewed in (Gipson, 2004)). However, only *MUC5AC*, and very low levels of *MUC2*, are found in the tears (Gipson, 2004).

Thirteen of the genes listed in Table 1 encode MAMs. *EMCN* is unusual in that it is an “endothelial mucin”, expressed primarily by vascular endothelial cells. The other twelve genes encode “epithelial mucins”, expressed by mucosal epithelia. Detection at the human

ocular surface of RNA transcripts and protein products encoded by *MUC1*, *MUC4*, *MUC16* and *MUC20* has been reported in published articles in the scientific literature.

Figure 1 shows a direct comparison by RT-PCR of MAM mRNAs accumulated at the apical surface of human conjunctival epithelium as sampled by impression cytology, a technique in which a supportive filter is pressed on the ocular surface and then removed along with adherent material. It was determined that *MUC20* is the gene most highly expressed in this location (Woodward and Argueso, 2014).

We report here, for the first time, that RNA transcripts and proteins encoded by the more recently characterized genes *MUC21* (Itoh et al., 2008) and *MUC22* (Hijikata et al., 2011) are also expressed at the human ocular surface (see below).

B. *MUC1*, *MUC4*, *MUC16*

MUC1 was originally called the polymorphic epithelial mucin or episialin, a glycoprotein identified at the cell surface of human mammary carcinomas (Gendler et al., 1990; Ligtenberg et al., 1990). High expression levels in tumors correlate with a poor patient prognosis due to its ability to promote cell growth and survival (Xu et al., 2015). The National Cancer Institute ranks *MUC1* as #2 in priority on a list of antigens for development as cancer vaccine targets, where major criteria are immunogenicity, oncogenicity and therapeutic function (Cheever et al., 2009).

MUC4 is the human homologue of rat sialomucin complex (SMC) (Moniaux et al., 1999), a high molecular weight glycoprotein heterodimer, originally discovered on the cell surfaces of the highly metastatic 13762 rat mammary adenocarcinoma (Carraway et al., 2000; McNeer et al., 1997). *MUC16* corresponds to the CA125 antigen (O'Brien et al., 2001; Yin and Lloyd, 2001), a well-studied human ovarian cancer marker (Bast et al., 1983; Meyer and Rustin, 2000).

Human genes for *MUC1*, *MUC4* and *MUC16* are located on chromosomes 1, 3 and 9, respectively (Table 1). These genes are expressed in epithelial cells that line the mucosal surfaces of many different tissues. In general, it was found that RNA is expressed throughout the epithelial layers, but the translated protein accumulates only in the most apical layer (discussed (Lomako et al., 2010)).

The difference between RNA expression and protein accumulation of *MUC1*, *MUC4* and *MUC16* has been clearly documented at the ocular surface. Thus, *in situ* hybridization has revealed *MUC1* mRNA in all cells of both corneal and conjunctival epithelia of humans (Gipson, 2000). However, immunoreactive *MUC1* protein can be detected only in apical cells of the corneal epithelium, and in apical and sub-apical cells of the conjunctival epithelium (Inatomi et al., 1995).

The available antibodies for rat Muc4 reacted against carbohydrate epitopes, and were non-specific in humans, so the localization in the epithelial layers has still not been determined in humans. However, the rat Muc4 antibody 15H10, stained only the superficial epithelial layers of rat corneal and conjunctival epithelia (Pflugfelder et al., 2000; Swan et al., 2002).

MUC16 protein was demonstrated in apical cells of corneal epithelia and in apical and sub-apical cells of conjunctival epithelia (Argueso et al., 2003).

Because many of the early antibodies used for immunolocalization were reactive only to MAM carbohydrate moieties, it has been conjectured that the apparently more restricted pattern of MAM protein localization as compared to mRNA expression might actually represent restricted glycosylation, and thus, restricted epitope expression. However, this explanation has not held up with newer studies using antibodies against MAM protein epitopes. A more likely explanation is regulation at the level of protein accumulation. In rat mammary gland epithelium, it was shown that Muc4 protein accumulation is restricted to the apical cell layer via a novel post-translational mechanism (Price-Schiavi et al., 2000). This was first defined in rat tumor cells, where it was shown that the proteasome degrades Muc4 (Swan et al., 2002). In stratified cultures of rat corneal epithelial cells, Muc4 protein levels are also regulated via the proteasome, which is apparently less active in the apical cell layer (Lomako et al., 2010).

The relative expression patterns of *MUC1*, *MUC4*, and *MUC16* mRNAs differ across the ocular surface epithelia. *MUC1* and *MUC16* mRNAs are homogeneously expressed across the corneal and conjunctival epithelia (Argueso et al., 2003; Gipson, 2000). However, *MUC4* mRNA is most abundant in conjunctival epithelium with an apparent diminution toward central corneal epithelium (Inatomi et al., 1996; Pflugfelder et al., 2000).

Of significance for inflammatory and autoimmune diseases of the ocular surface, *MUC1* is also expressed by immune cells that are resident in the ocular surface epithelial, or that infiltrate due to inflammatory or immunological events, including B cells, T cells, monocytes, macrophages and dendritic cells (Agrawal et al., 1998; Brugger et al., 1999; Leong et al., 2003; Wykes et al., 2002).

In addition to apical epithelial cells of the ocular surface, MUC16 was immunolocalized to goblet cells of the conjunctiva, associated with the goblet cell mucin granule membrane (Gipson et al., 2016). A similar localization to goblet cell mucin granules is seen in the respiratory epithelium, as well as mucus cells in the submucosal gland (Davies et al., 2007; Kesimer et al., 2013).

MUC1, *MUC4* and *MUC16* are also expressed in the lacrimal gland (Jager et al., 2007; Jumblatt et al., 2003; Paulsen et al., 2004). A particularly detailed study of MUC16 was performed (Jager et al., 2007). MUC16 immunoreactivity was associated with the plasma membrane in accessory lacrimal glands. In the main lacrimal gland, as well as acinar cells and columnar cells of the nasolacrimal ducts, MUC16 immunoreactivity was also detected in intracytoplasmic vesicles. Subepithelial serous glands of the nasolacrimal ducts were also stained. Reactivity was further visible in secretion products within the lumen of serous acini and the nasolacrimal passage.

Considering that MAMs are membrane-tethered, the location of MUC16 within secreted lacrimal gland fluids seems contradictory at first. However, many of the MAMs can be immunodetected as soluble forms in the various extracellular fluids of the body (Moniaux et al., 2001). Early papers conjecture about whether this is due to their secretion. However, it

is now known that part of the extracellular component of the MAM is shed from mucosal epithelial surfaces into extracellular fluids (discussed more in Section III). Consistent with this, immunoreactivity for MUC1, MUC4 and MUC16 has been detected in human tear fluid (Spurr-Michaud et al., 2007). Thus, the mucin component of tears is primarily a mixture of the secreted mucin MUC5AC and the soluble shed subunits of MUC1, MUC4, and MUC16.

In tracheobronchial, gastrointestinal and reproductive tracts, gel-forming mucins are secreted from goblet cells to create a viscous mucous layer which spreads over the epithelial glycocalyx. Imaging studies of fixed tissues have suggested that a distinct mucous layer is also associated with the epithelial glycocalyx. This is overlain by the aqueous component of tears, which is surfaced by lipid (Holly and Lemp, 1977; Johnson and Murphy, 2004; Nichols et al., 1985). However, a study utilizing biophysical methodologies of unfixed tissues has questioned the idea of distinct layers (Hodson and Earlam, 1994). In mice, studies employing electron microscopy following *in vivo* cryofixation with freeze substitution revealed a homogenous, fine network-like structure throughout the tear film, consistent with a model of mucins suspended in the aqueous phase (Tran et al., 2003). It is now generally accepted that the aqueous and mucin components of the tears combine to create a single layer of mucoaqueous gel (Willcox et al., 2017).

The single-phase model of the tear film makes functional sense, as a distinct layer of thick, light-scattering mucus on the surface of the cornea would obscure the central visual axis. Further to this idea, MUC5AC in tear fluid was shown to have an increased electrophoretic mobility compared to MUC5AC isolated from conjunctival tissue (Berry et al., 2004; Spurr-Michaud et al., 2007) and there is evidence that MUC5AC can be cleaved (Lidell and Hansson, 2006). A smaller size mucin molecule might facilitate the mixing of the aqueous and fluid components of tears.

With their complement of highly glycosylated mucins, the tears are hydrophilic and hygroscopic, maintaining fluid on the ocular surface, resisting drainage and contributing to lubrication of epithelial surfaces to limit frictional damage (Mantelli and Argueso, 2008). The tears move easily between the lid and over the glycocalyx because the mucins of both have anionic character that creates repulsive forces between them (Gipson, 2004). The tear mucins are also believed to trap and remove surface debris through movement over the ocular surface (Gipson and Inatomi, 1998).

C. MUC20

MUC20 was identified in a screen for genes with altered expression in renal tissues of patients with immunoglobulin A nephropathy (Higuchi et al., 2004b). It is localized at human cytogenetic locus 3q29, just upstream of *MUC4*. The two genes lie “head-to-head”, being transcribed in opposite directions. Directly upstream of *MUC20* is *MUC20P1*, a gene fragment related to *MUC20* by sequence. Classified as a pseudogene, it seems likely that *MUC20P1* arose through a duplication of a portion of *MUC20*.

In human eyes, immunoreactive MUC20 was detected throughout the entire ocular surface epithelia, but predominantly within the plasma membrane region of intermediate cell layers. MUC20 also was observed in the cytoplasm of apical cells within the stratified squamous

epithelium of the conjunctiva, but not in goblet cells (Woodward and Argueso, 2014). It was not found in tears. Thus, MUC20 exhibits a different localization pattern as compared to the other expressed MAMs.

D. MUC21 and MUC22

MUC21 and *MUC22* came to our attention during a genome-wide association study to identify single nucleotide polymorphisms (SNPs) associated with steroid-induced ocular hypertension (Jeong et al., 2015). Figure 2 shows the position of the two genes, located adjacent to one another at human chromosomal locus 6p21.33, both transcribed in the same direction. This genomic region lies within the major histocompatibility complex (*MHC*) on chromosome 6. *MUC21* was identified by homology search using a cDNA fragment encoding mouse epiglycanin, a cell surface glycoprotein expressed by a subline of TA3 mouse mammary carcinoma cells. Analysis using tissue cDNA libraries indicated that the gene is expressed in numerous mucosal tissues, including lung, large intestine, thymus and testis (Itoh et al., 2008).

MUC22 was identified as part of a study on diffuse panbronchiolitis (DPB), a rare complex genetic disease of the respiratory system. An HLA-associated major susceptibility gene for DPB was located within the 200 kb in the class I region 300 kb telomeric of the HLA-B locus on chromosome 6. Within this candidate region, a novel mucin gene was identified, located adjacent to *MUC21*. *MUC22* expression was examined by PCR screening of a commercial human multiple tissue cDNA panel. Expression was detected in the mucosal tissues of lung, placenta and testis (Hijikata et al., 2011). *MUC22* mRNA expression was also detected in a tissue-equivalent model of primary human bronchial epithelial cells. Immunolocalization analysis in lung identified *MUC22* antibody staining within the cytoplasm of serous cells of the submucosal gland. The submucosal glands are responsible for secretion of the periciliary liquid, which is analogous to the tear fluid secreted by the lacrimal gland. It is important for mucous clearance in the airways (Sharma et al., 1998).

Figure 2 shows expression analysis of *MUC21* and *MUC22* at the mRNA level by RT-PCR in three different cell types of the anterior segment: 1) a trabecular meshwork cell line, 2) primary cultures of trabecular meshwork cells, and 3) primary cultures of human corneal epithelial cells in monolayer culture. Both genes were expressed by the corneal epithelial cells. This figure was previously published (Jeong et al., 2015).

Figure 3 depicts previously unpublished results of *MUC21* and *MUC22* immunolocalization in the human corneal epithelium and lacrimal gland. Formalin-fixed, paraffin-embedded tissues were cross-sectioned, then the sections were processed and indirectly immunostained as described (Itakura et al., 2019). A 3,3'-diaminobenzidine (DAB) chromogen kit was used to detect secondary antibody binding. The affinity-purified *MUC21* primary antibody was derived from a rabbit polyclonal antisera raised against a peptide from the human *MUC21* cytoplasmic tail. The affinity-purified *MUC22* antibody was characterized in one of our labs (Hijikata et al., 2011). It was derived from a rabbit polyclonal antisera raised against a peptide from the human *MUC22* extracellular domain.

Both MUC21 and MUC22 antibodies stained cells of the apical layer of human corneal epithelia. Both also stained specific cells in the lacrimal gland, but the pattern for each was different.

Muc21 antibody staining of lacrimal gland was intense in the epithelial cells lining the lacrimal ducts. Staining appeared to be both membranous and cytoplasmic. Only the occasional acinus was stained; again, staining was both membranous and cytoplasmic.

In contrast, MUC22 antibody primarily stained the serous acini. Staining was cytoplasmic (much as seen in the serous cells of the lung submucosal gland) and was concentrated within intracytoplasmic vesicles. Epithelial cells lining some lacrimal ducts were also stained with the MUC22 antibody, although fewer of these than for MUC21, and staining was both cytoplasmic and membranous. In some cases, immunoreactive material was observed within a duct.

This analysis identifies, for the first time, two new genes expressed at the ocular surface, as assessed by the dual criteria of RT-PCR (mRNA) and immunolocalization (protein). The protein products of both genes are also localized to the lacrimal gland.

III. Structure/Function

A. General

1. Length and Conformation—The MAMs are the largest of the membrane-associated glycoproteins. Table 2 ranks human MAMs by the length of their polypeptide chain. The longest MAM, MUC16, is close to 15,000 amino acids. The clustering of O-linked oligosaccharide chains within the tandem repeats creates steric interactions between carbohydrate and peptide, inducing the peptide core to adopt a stiff and extended conformation. This results in projection of the MAM well above the cell surface, far beyond other membrane-associated proteins (Jentoft, 1990). MAMs would, therefore, be the first molecules encountered by invading pathogens, and are thus positioned to shield and protect the cell surface.

Table 2 gives a count for the number of isoforms of each MAM listed in the NCBI Gene database. This includes splice variants as well as variants with insertions and deletions. It is important to note that genomic information such as this, is a work in progress. For example, while gathering information for the table, we observed a large discrepancy between the length of the MUC1 protein isoforms currently represented by NCBI (which top out at 484 amino acids) and the canonical isoform P15941–1 of 1255 amino acids in length, as represented by UniProt (<https://www.uniprot.org/help/about>). This was perplexing, as published articles describe the longer form (Bafna et al., 2010).

In consulting with NCBI staff, we learned that P15941–1 is based on a mRNA, J05582.1, that was cloned from a pancreatic tumor (Lan et al., 1990). NCBI does not usually list variants from cancer tissues, as they may be unique to the specific tumor. In fact, J05582.1 aligns poorly to the current human genome assembly, suggesting this is the case. However, it was noted that alignment of other mucin genes in the mouse and human genomes has

also been difficult due to their high sequence repeat content, suggesting that it is equally possible the alignment problem lies on the genome side. This is being reported to the Genome Reference Consortium so that it might be revisited in the next human assembly update (personal communication, Dr. Eric Cox, NCBI).

2. Biophysical Properties—As noted in the Introduction to this article, the tandem amino acid repeat unit, densely modified by O-linked glycan chains, is the distinguishing feature of MUC family mucins. The number of tandem repeats can vary considerably among individuals within a population, leading to the designation of “VNTR” for variable number of tandem repeats (Gendler and Spicer, 1995). This variation accounts for some of the isoforms of a given MAM listed in Table 2; many more have been identified in cancer cells.

The extensive glycosylation of the numerous serine and threonine residues within tandem repeat regions, confers a hydrated, hydrophilic character (Argueso and Gipson, 2001). The densely-packed glycan chains also confer an antiadhesive character to cell surfaces. In cultured cancer cells, overexpression of MAMs stimulates cell detachment from their substratum, which is more pronounced the greater the number of tandem repeats (Berry et al., 2001). MAMs were also shown to confer a disadhesive character to the apical surface of corneal epithelial cells, suggesting that they provide boundary lubrication and prevent adhesion of facing cell surfaces (i.e. corneal epithelium and tarsal conjunctiva) during blinking or sleeping (Sumiyoshi et al., 2008).

The glycan moieties may vary depending on the mucin type, the site of mucin expression, and the physiological or pathological conditions (Chaturvedi et al., 2008). Abnormalities in MAM O-glycosylation have been identified in many disorders where the stability of the tears is compromised, such as contact lens wear and dry eye (e.g., (Gipson et al., 2004); reviewed in (Guzman-Aranguez and Argueso, 2010)). When the glycocalyx is altered pathologically, wetting of the cornea becomes imperfect and tear stability is compromised.

3. Cell Surface Receptor—In addition to the functions conferred by the O-linked glycan chains, it is increasingly appreciated that MAMs also serve as cell surface receptors that sense the extracellular environment and transduce signals intracellularly. This has been studied primarily using monolayer cultures of various types of cancer cells. In these cells, MAMs have been shown to activate or inhibit intracellular signaling cascades that regulate inflammation, cell-cell interactions, differentiation and apoptosis (Constantinou, 2011; Hollingsworth and Swanson, 2004; van Putten and Strijbis, 2017).

Figure 4 depicts a prototypical MAM, the structure of which is similar to a classic, single-pass transmembrane immune receptor. A signal peptide motif is found at the N-terminal of the precursor polypeptide chain to enable its membrane insertion; it may be retained in the mature protein (1). The mature protein is composed of two subunits that self-associate, arising from intracellular cleavage. The large subunit is entirely extracellular and contains the VNTR. The small subunit consists of a short extracellular region, a single-pass transmembrane domain and a cytoplasmic tail (CT).

The large subunit of the MAM, together with the extracellular portion of the small subunit, comprise the extracellular domain (ED). Besides the VNTR, with its sites for O-linked glycosylation, a number of sites for N-linked glycosylation are interspersed across the ED. The ED also contains conserved sequence motifs as modular elements that are mixed and matched in each MAM.

The Sperm protein, Enterokinase and Agrin module (SEA) and EGF-like modules are found in multiple MAMs and are shown on the MAM prototype in Figure 4. Other conserved sequence modules specific to individual MAMs are listed in Table 3. Most are located in the ED. These modules participate in signal transduction, as discussed in the next subsection.

As mentioned briefly in Section II of this article, many of the MAMs can be immunodetected as soluble forms in the various extracellular fluids of the body, and are also found in the extracellular media of cultured cells (Moniaux et al., 2001). This is the result of “shedding” of the large subunit of the MAM as it projects from the cell surface. Shedding may occur spontaneously, but can be stimulated by binding of the large subunit to bacteria and other ligands. A number of biologically important proteins bind the carbohydrate side-chains of MAMs, including galectins, selectins and siglecs (sialic acid-binding immunoglobulin-type lectins)(Bochner and Zimmermann, 2015). Cytokines and extracellular proteinases also promote shedding, as do a variety of physical conditions such as mechanical force or changes in pH, ionic concentration or degree of hydration (Albertsmeyer et al., 2010; Hollingsworth and Swanson, 2004). Shedding may be one stimulus initiating signal transduction.

Table 4 lists CTs in epithelial MAMs, ranked by length. The CTs are quite short in comparison to the EDs. Strikingly (but of unknown significance), the two longest MAMs (MUC16 and MUC4) have considerable shorter CTs than the others. The CTs of individual MAMs are dissimilar in sequence and length and do not contain conserved domains (except for MUC21 and MUC22). Ligand binding, shedding of the large subunit, or other external stimuli leads to engagement of receptor tyrosine kinases or other protein kinases and phosphorylation at specific tyrosine or serine/threonine residues in the CT. Phosphorylation initiates signal transduction cascades that regulate inflammation, cell-cell interactions, differentiation and apoptosis (Bafna et al., 2010; Kato et al., 2012).

4. Evolution—Other than the serine and threonine residues needed for O-linked glycosylation, the amino acid sequences and length of the tandem repeats differ among the different MAMs. The lack of sequence similarity suggests that the MAMs independently evolved the VNTR. In evolutionary biology, organisms not closely related, can independently evolve similar traits as a result of having to adapt to similar environments or ecological niches. This process is called convergent evolution.

On the other hand, evaluation of the shared modules has revealed evolutionary relationships among the MAMs (Dekker et al., 2002). For example, *MUC1* has no sequence similarity with the other MAMs except for the presence of the SEA module. This module originated from HSPG2 (perlecan). The *MUC1* SEA module is most closely related to those found in *MUC3*, *MUC12* and *MUC17* (all grouped at chromosomal locus 7q22.1) as well as *MUC13*

(at 3q21.2). *MUC13* appears to be the ancestral gene, with the others likely the result of duplicative events in evolution. *MUC1* (at 1q22) may have lost the two EGF-like modules found in the other subfamily members after its duplication (Duraismy et al., 2006).

In contrast, *MUC16* SEA modules are most-closely related to the SEA module found in the chicken *AGRN* (*agrin*) gene. The prototype of this SEA module appears to have evolved before the divergence of birds and mammals (Duraismy et al., 2006).

MUC4 has a number of modules not found in other MAMs. The NIDO module evolved from an ancestor common to the NIDO (*nidogen*) protein, and the AMOP and VWD modules originated from an ancestor common to the Sushi-domain containing proteins (Duraismy et al., 2006).

MUC20, which lies adjacent to *MUC4* at chromosomal locus 3q29, also lacks these modules. However, analysis using the EMBL-EBL multiple sequence alignment tool, Clustal Omega (<https://www.ebi.ac.uk/Tools/msa/clustalo/>) indicates significant sequence similarity between the *MUC4* VNTR and the entire length of *MUC20* (S. Jeong, previously unpublished data). This strongly suggests that *MUC20* arose by duplication of the *MUC4* VNTR followed by sequence divergence.

The most recently discovered *MUC21* and *MUC22*, clustered together at chromosomal locus 6p21.33, also do not share motifs in common with the other MAMs. The VNTR of *MUC21* is recognized as a conserved motif called Epiglycanin_TR (Table 3) that is shared by genes of two lower species. The VNTR of *MUC22* is not part of this family. However, *MUC21* and *MUC22* share sequence similarity with one another through the Epiglycanin_C domain, which covers the region downstream of the VNTR, including a putative cleavage site, the transmembrane domain and the CT (Table 3). The presence of this domain provides evidence that *MUC21* and *MUC22* are the result of an evolutionary duplication event.

Figure 5 depicts the suggested evolutionary relationships among the epithelial MAMs. This analysis supports the concept that MAMs arose largely through a process of convergent evolution, but reveals that they can be grouped into evolutionarily-related subgroups based on their genetic backgrounds. Within a group, evolutionary duplicative events appear to have led to divergent evolution, the process whereby groups from the same common ancestor accumulate differences to serve specific purposes.

B. MUC1, MUC4 and MUC16

1. The Extracellular Domain—Figure 6 depicts the modular architecture of the EDs of *MUC1*, *MUC4* and *MUC16* (as well as *MUC20*, *MUC21* and *MUC22*, to be discussed later).

The tandem repeats in the VNTR of *MUC1* are 20 amino acids in length, with 25 to 125 repetitions. In *MUC4* they are 16 amino acids in length, with 145 to 395 repetitions. *MUC1* contains one SEA module, located just proximal to the transmembrane domain. *MUC4* lacks a SEA module, but has modules for conserved motifs AMOP, NIDO, and VWD, clustered together, distal to the VNTR. Flanking the VNTR in *MUC1* are two regions of unique

sequence that are serine and threonine rich and heavily O-glycosylated, like the VNTR. Similarly, MUC4 has a region of imperfect tandem repeats proximal to the tandem repeat unit, also heavily-glycosylated.

The VNTR of MUC16 is unusual in that it contains long, only partially conserved tandem repeat units of 156 amino acids. Variants of this gene encode proteins with 10 to 60 repeats (NCBI Gene); the variant listed in Table 2 has a shorter VNTR than reported in (O'Brien et al., 2001; O'Brien et al., 2002). Proximal to the VNTR is a long region of unique sequence (12,070 amino acids) that is serine and threonine rich and (like the VNTR) is heavily O-glycosylated. Interspersed in the VNTR, and distal to it are a total of 56 SEA modules. The VNTR also contains interspersed leucine-rich repeats and ankyrin repeats (not shown). Each of these features contribute to the very long ED of MUC16 (Perez and Gipson, 2008).

It has been estimated that an extended, O-glycosylated polypeptide of 20 amino acid residues is approximately 5 nm long (Jentoft, 1990). This would mean that human MUC1 extends about 200 – 500 nm above the cell surface. MUC4 would extend at least 2 microns and MUC16 could be twice that, at 4 microns.

During its biosynthesis, MUC1 is cleaved within the SEA module (Palmai-Pallag et al., 2005), while MUC4 is cleaved within the juxtamembrane VWD module (Rossi et al., 1996). This processing occurs in the endoplasmic reticulum after N-glycosylation (Ligtenberg et al., 1992). The complex then migrates to the Golgi apparatus, where it is O-glycosylated, and finally moves to the cell surface, where the two subunits remain strongly associated via non-covalent interactions. It was long speculated that MUC16 harbors single sites for proteolysis in each of the two SEA modules adjacent to the plasma membrane, one being analogous to the MUC1 site. However, it was shown recently that actual cleavage takes place in the juxtamembrane ectodomain stretch of twelve amino acids, and occurs within the Golgi/post-Golgi cellular compartment (Das et al., 2015).

Cleavage of MUC1 within the SEA module also can occur extracellularly via the proteolytic action of ADAM17 (Thathiah et al., 2003) or MT1MMP (Thathiah and Carson, 2004). MUC16 is cleaved extracellularly by proteases such as MMP7, ELNE (neutrophil elastase) and bacterial metalloprotease (ZmpC), although the exact site(s) is not known (Blalock et al., 2008; Govindarajan et al., 2012). Extracellular cleavage results in enhanced shedding of the large subunit from the cell surface.

The ED of MUC4 (but not MUC1 or MUC16) has three EGF-like modules located distal to the cleavage site (Hanson and Hollingsworth, 2016). The one closest to the transmembrane domain is similar in sequence to the EGF-like domain found in ERBB3, a receptor tyrosine kinase of the EGFR family. Rat Muc4 was shown to interact via this EGF-like module with ERBB2, another member of the family. The protein-protein interaction induced specific phosphorylation of ERBB2 and led to downstream signaling (Jepson et al., 2002). Complex formation also potentiated activity of ERBB3 stimulated by binding to NRG1 (neuregulin). This implicated Muc4 in regulation of epithelial cell proliferation in rat carcinoma.

2. The Cytoplasmic Tail—Figure 7 depicts an alignment the CTs of human, mouse and rat MUC1, MUC4 and MUC16, delineating, in red, experimentally confirmed serine, threonine or tyrosine phosphorylations, as curated by the public database PhosphoSitePlus® (Hornbeck et al., 2019). Some of the known MUC1 CT interacting proteins are indicated in red above their recognition sequences. Sites predicted to be phosphorylated by analysis using the NetPhos 3.1 Server (Blom et al., 2004) are delineated in blue, along with predicted protein kinase effector (M.E. Fini, previously unpublished). Polybasic amino acid stretches previously identified in human are highlighted in yellow for all three species.

The CT of MUC1 is the best studied by far. It has been observed that the amino acid sequence is highly conserved across species (Spicer et al., 1991; Vos et al., 1991), as demonstrated here for human, mouse and rat (Figure 7). Seven tyrosines and eleven serines/threonines have been experimentally confirmed to be phosphorylated under various conditions. Four of the confirmed tyrosine phosphorylations are located within sequences that constitute signaling protein binding motifs: Y²⁰HPM (phosphatidylinositol 3-kinase regulatory subunit PIK3R1); Y³⁵VPP (phospholipase PLCG1); Y⁴⁶EK/EV (SRC family kinases); Y⁶⁰TNP (GRB2) (Zrihan-Licht et al., 1994). Molecular and biological effects have been determined for all four of these tyrosines as well as two of the serine/threonines.

In just one example, EGFR, a receptor tyrosine kinase of the same family as ERBB2, associates constitutively with the MUC1 CT in human breast carcinoma cells. Active EGFR binds the MUC1 CT at Y⁴⁶EK/EV and phosphorylates the tyrosine residue (Schroeder et al., 2001). This phosphorylation stimulates SRC binding (Li et al., 2001). SRC strengthens the binding of CTNNB1 (beta-catenin) to the sequence motif SAGNGGSSL by phosphorylating a different tyrosine residue located near the CTNNB1 binding site (Li and Kufe, 2001; Li et al., 2001). Conversely, binding of CTNNB1 is weakened by the activity of GSK3B, which binds the SXXXS motif located proximal to the CTNNB1 binding site, phosphorylating the final serine (Li et al., 1998). These effects on CTNNB1 binding strength, influence the ability of CDH1 (E-cadherin) of the adherens junction (Huang et al., 2005) to compete for binding to CTNNB1. This competition affects epithelial cell-cell adhesion (Quin and McGuckin, 2000; Yamamoto et al., 1997).

Much less is known about the MUC16 CT however, it appears that EGFR-mediated phosphorylation may occur here as well. MUC16 contains two tyrosine residues and one serine residue confirmed experimentally to be phosphorylated (Figure 7). An analysis conducted using the NetPhos 3.1 Server (Blom et al., 2004) predicts that EGFR phosphorylates the proximal tyrosine residue (M.E. Fini, previously unpublished). Large subunit shedding stimulates CT phosphorylation and this is enhanced when cells are treated with EGF (Fendrick et al., 1997).

Also like MUC1, MUC16 binds to CTNNB1-CDH1 complexes (Comamala et al., 2011). MUC16 lacks a canonical CTNNB1 binding site, but pull-down experiments suggest that a polybasic amino acid stretch at the proximal end of the CT interacts with ezrin/radixin/moesin (ERM) actin-binding proteins that then interact with the adherens junction (Blalock et al., 2007). NetPhos 3.1 Server also predicts that MUC16 is phosphorylated by CDK1. In complex with CCNA2 (cyclin A2), CDK1 promotes adhesion complex and

actin cytoskeleton organization during interphase and mediates a large increase in adhesion complex area as cells transition from G1 into S (Jones et al., 2018).

The CT of many cell surface receptors migrates to the nucleus to perform additional functions, often initiated by shedding of the large subunit. This has been shown to occur in the case of both MUC1 and MUC16. MUC1 traffics to the nucleus in complex with CTNNB1, raising the possibility that MUC1 might directly influence the transcriptional co-activator activity of CTNNB1 (Li and Kufe, 2001; Ren et al., 2002). Nuclear translocation appears to involve endocytosis of MUC1 from the cell surface as a first step, requiring phosphorylation of Y⁶⁰TNP and binding of GRB2 (Kinlough et al., 2004).

Essentially nothing is known about function of the MUC4 CT. The amino acid sequence is poorly conserved between humans and mouse/rat, with only three amino acids conserved among the three species (Figure 7). An analysis of the human sequence conducted using the NetPhos 3.1 Server predicts phosphorylation at three different serine residues by protein kinase A and protein kinase C isoforms (M.E. Fini, previously unpublished). Interestingly, two of these serines are conserved in the CT of mouse and rat and are also predicted to be phosphorylated. The CT of mouse/rat are slightly longer than human, and possesses an additional predicted site for phosphorylation site by the catalytic subunit of casein kinase II, CSNK2A1. Interestingly, it has been reported that CSNK2A1 interacts with the adherens junction and modulates intracellular adhesion (Lickert et al., 2000).

C. MUC20

Structural architecture of the MUC20 ED is depicted in Figure 6. The predicted human MUC20 isoform NP_001269435.1 is a polypeptide of 709 amino acids with a signal peptide of 22 amino acids. All four isoforms currently listed in NCBI's database have predicted signal peptides (a previous report discussed a variant lacking a signal peptide (Higuchi et al., 2004b)). Isoform NP_001269435.1 has 12 tandem repeat units of 19 amino acids each. The other isoforms have three or four tandem repeats. Extensive O-linked glycosylation of the tandem repeats is predicted by sequence analysis using the NetOGlyc 4.0 Server (Steentoft et al., 2013) (M.E. Fini, previously unpublished). MUC20 lacks SEA or EGF-like modules. However, it contains one copy of a conserved domain, PHA03247, which is also found in the large tegument protein of *Herpes simplex* virus type I (Table 3). This module follows the tandem repeats.

Sequence analysis of human MUC20 identified several hydrophobic regions consistent with plasma membrane association, but no alpha-helical transmembrane domain was recognized (Higuchi et al., 2004b). Application of the TMPred tool (Hofmann and Stoffel, 1993) on the ExPASy Bioinformatics Resource Portal (Artimo et al., 2012) did not identify a transmembrane domain (M.E. Fini, previously unpublished). Nevertheless, when MDCK cells harboring a human *MUC20* expression construct were biochemically fractionated, MUC20 protein was identified in the membrane fraction, which includes plasma membrane, endoplasmic reticulum and golgi. Immunoelectron microscopic analysis of whole cells demonstrated localization to the plasma membrane (Higuchi et al., 2004b).

In a second study from the same authors, MUC20 immunoreactivity was observed in the basal membranes of proximal tubular epithelia of the human kidney (Higuchi et al., 2004a). In human ocular surface epithelia, immunoreactive MUC20 was detected predominantly in the cell membrane area of intermediate cell layers (Woodward and Argueso, 2014). Biotin labeling of the surface of corneal epithelial-equivalent cultures revealed only low levels of MUC20 protein on apical glycocalyxes.

Thus, current evidence suggests MUC20 is a non-secreted protein retained at the plasma membrane, but possibly extrinsically rather than transmembrane. For this reason, only the MUC20 ED is depicted in Figure 6, and not the distal regions. In the ocular surface study discussed above, MUC20 was not detected in the media of epithelial tissue-equivalent cultures or in human tears, consistent with the idea that it is neither secreted nor shed (Woodward and Argueso, 2014).

Because of uncertainty about its structure, MUC20 is not included in the analysis of CT sequences shown in Figure 6. Analysis, using the NetPhos 3.1 Server, of the short amino acid sequence following a predicted alpha-helical region near the C-terminus of MUC20 predicted no potential phosphorylation sites of statistical significance (M.E. Fini, previously unpublished). Recombinantly-expressed human MUC20 was shown to associate via its C-terminal domain with MET, a receptor tyrosine kinase activated by the extracellular ligand HGF. The interaction prevented GRB2 recruitment to MET, attenuating HGF-induced activation and intracellular signaling (Higuchi et al., 2004a).

D. MUC21 and MUC22

The modular architecture of MUC21 and MUC22 is depicted in Figure 6.

The human MUC21 protein predicted by NCBI Protein entry NP_001309299.1 is the longest of three variant isoforms listed in the NCBI Gene database, and its analysis has not previously been described. The NCBI Protein profile of the variant predicts a signal peptide of 24 amino acids, followed closely by a series of 32 imperfect tandem repeat units of 15 amino acids each. Extensive O-linked glycosylation within the tandem repeat units was predicted for another variant (Itoh et al., 2008) by sequence analysis using the NetOGlyc 4.0 Server (Steentoft et al., 2013). The TMPred tool (Hofmann and Stoffel, 1993) on the ExPASy Bioinformatics Resource Portal (Artimo et al., 2012), predicts an alpha-helical transmembrane region of 21 amino acids, followed by a CT of 59 amino acids (M.E. Fini, previously unpublished).

Analysis of the human MUC22 protein predicted by NCBI Protein NP_001185744.1 has previously been reported (Hijikata et al., 2011). It has an N-terminal signal peptide of 26 amino acids. The ED contains 124 non-identical tandem repeats of 10 amino acids each. Extensive O-linked glycosylation of the tandem repeats is predicted by the NetOGlyc 4.0 Server (Steentoft et al., 2013) (S. Jeong, previously unpublished). Following this is an alpha-helical transmembrane domain of 21 amino acids and an CT of 92 amino acids.

It is not known whether MUC21 or MUC22 are cleaved during their biosynthesis. Both MUC21 and MUC22 lack SEA modules, however, a sequence (GSLV) similar to the

putative cleavage site associated with the SEA module in MUC1 is present immediately upstream of the putative transmembrane domain in MUC21 (Itoh et al., 2008). This potential cleavage site is included in the conserved motif Epiglycanin_C, domain shared by MUC22, but the specific sequence is not conserved.

Function of the MUC21 tandem repeats in cell adhesion to the substratum was investigated by transient transfection analysis (Yi et al., 2010). When HEK 293T cells were transfected with a mouse Muc21 expression construct harboring a cDNA containing 84 tandem repeat units, cells were significantly less adherent to each other and to extracellular matrix components than control cells. The anti-adhesion effect was weaker when constructs with smaller numbers of tandem repeats were used, suggesting that the tandem repeat domain plays a crucial role. Antibody binding to the cell surface integrin subunits ITGA5, ITGA6, and ITGB1 was reduced in MUC21 transfectants in a tandem repeat-dependent manner, whereas equal amounts of proteins were detected by Western blot. MUC21 was expressed as a large glycoprotein that was highly glycosylated with O-glycans at the cell surface, as detected by flow cytometry, Western blotting, and lectin blotting. Although at least a portion of Muc21 was glycosylated with sialylated glycans, removal of sialic acid did not influence the ant-adhesive effect.

The MUC21 and MUC22 CTs are depicted in Figure 7. MUC21's CT is of moderate length as compared to the other MAMs. At 92 amino acids, MUC22's CT is the longest of all the MAMs expressed at the ocular surface. According to the PSIPRED Workbench (Buchan et al., 2013) tool for predicting protein secondary structure (Jones, 1999), each CT likely contains specific regions of disordered structure, providing elements for binding of other proteins (S. Jeong, previously unpublished).

MUC21's CT is characterized by the presence of seven proline residues. The proline-rich regions are known to preferentially adopt a polyproline type II helical conformation, an extended structure that facilitates transient intermolecular interactions important to intracellular signaling (Srinivasan and Dunker, 2012). For example, many adapter proteins possess specific protein domains such as the Src homology 3 (SH3) domains and the WW domains that selectively recognize proline-rich regions in their interacting partners (Mansiaux et al., 2011; Peterson and Volkman, 2009).

MUC22's CT is rich in glycine, which comprises more than 1/4th of the total amino acid residues (26/92). Glycine is unique in that the side chain consists of only a single hydrogen atom, providing for flexible conformation. There is also an enrichment for His, which is unusual in having a PKa of 6.5, ~physiological pH. This means it exists simultaneously in protonated/deprotonated forms, a feature that could activate binding proteins. Two cysteine residues are located at the proximal aspect of the CT as it emerges from the plasma membrane, providing for possible internal disulfide coupling, or coupling with other proteins.

As will be discussed more in Section IV, neither a laboratory rat orthologue to human MUC21, nor mouse or rat orthologues to human MUC22 has been identified. The amino acid sequence of the MUC21 CT is poorly conserved between human and mouse. One

site for tyrosine phosphorylation and three sites for serine/threonine phosphorylation have been experimentally confirmed in the human MUC21 CT. Significantly, all four sites are conserved in mouse. A single tyrosine and three serine phosphorylation sites in the MUC22 CT are predicted by the NetPhos 3.1 Server (Figure 7).

EGFR is predicted to phosphorylate the tyrosines in the CTs of both MUC21 and MUC22. The serine/threonine sites are predicted to be phosphorylated by a member of the protein kinase A family and CDK1/CDK5. Activated by cAMP, PKA lies downstream of G protein-coupled receptors (GPCRs) that couple with G's. Thus, any ligand that activates these GPCRs should also activate MUC21 and MUC22. As noted with regard to MUC16, CDK1 promotes adhesion complex and actin cytoskeleton organization during interphase (Jones et al., 2018). Similarly, CDK5 has been shown to promote the stability of corneal epithelial cell junctions (Arpitha et al., 2013).

IV. Model Systems and Biological Roles

Human studies of the ocular surface are limited to non-invasive techniques such as tear collection and analysis, staining of the ocular surface with vital dyes, and impression cytology for collection of apical cells of the conjunctiva. Biopsies routinely taken in other organs such as skin are not done in cornea because of the resulting pain and disruption of vision. Human cadaver corneas obtained from a local eye bank or from the National Disease Research Interchange (Philadelphia, PA) can be placed in organ culture for study and are amenable to genetic manipulation. This has been an effective model for wound healing studies (e.g., (Castro et al., 2019; Kramerov et al., 2016)), however, the ocular surface glycocalyx is easily damaged in the Optisol storage medium (Chiron Vision, Claremont, CA) that is typically used by the eye and tissue banks.

Human tissue-equivalent and mouse models enable genetic manipulation, and have successfully substituted for investigation of MAM functional roles in the ocular surface mucosal glycocalyx. We discuss these models here. We go on to discuss key findings made using each. Individually and together these models have led to significant advances in our understanding of MAM roles at the ocular surface in health and disease, and hold much promise for new discovery.

A. Human Tissue-Equivalent Model

As described in the first paragraph of our Introduction, cells at the ocular surface are very different from the basal cells of the multilayered epithelia from which they arise. Significantly, while monolayer cultures of corneal or conjunctival epithelial cells express MAMs at the mRNA level, MAM proteins do not accumulate to detectable levels in these cultures. However, MAM proteins accumulate in a polarized manner at the surface of three-dimensional mucosal epithelial tissue-equivalents.

Human tissue-equivalents represent the human ocular surface glycocalyx with substantial fidelity. This model lacks *in vivo* complexity, for example, there is no immune system contribution. However, its reductionist nature provides an advantage for the study of

molecular pathway(s), as well as for isolating the contributions of individual tissues to complex biological responses.

Epithelial tissue-equivalent technology was developed first for skin. A technique for successful serial cultivation of epidermal keratinocytes was reported in 1975 by Rheinwald and Green (Rheinwald and Green, 1975). This involved plating dissociated cells on a feeder layer of mouse 3T3 fibroblasts that had been previously irradiated to preclude their proliferation. Cells of the feeder layer secrete soluble factors into the culture medium, and also deposit extracellular matrix on the culturing surface, facilitating keratinocyte cell attachment and growth, and enabling the clonogenic expansion of individual cells (Green et al., 1977). Stratification is then induced by increasing the calcium concentration and by “airlifting”, i.e., reducing the volume of culture medium so that the keratinocytes were located to the air–medium interface. Under these conditions, proliferating basal cells remain in close proximity to the gradient of nutrients provided by diffusion (Bernstam et al., 1986; Prunieras et al., 1983).

In recent years, defined media such as Keratinocyte Growth Medium[®] (Lonza, Walkersville, MD) and Epilife[®] (Invitrogen, Carlsbad, CA) have become commercially-available for serial culture and differentiation to an epidermal- or corneal epithelial-equivalent without the use of feeder layers (Argueso and Gipson, 2012; Rasmussen et al., 2013).

Human corneal epithelial-tissue equivalents typically made use of primary cells isolated from corneal–limbal rims discarded at the time of corneal transplantation. These usually contain tissue-specific stem cells. However, the finite replicative lifespan of the amplified progeny cells makes them impractical for research approaches requiring stable genetic transfection or genome modification. For such experiments, immortalized epithelial cell lines that retain differentiation characteristics have become widely used.

Corneal cell lines have been developed by immortalization with viral oncogenes, including adenovirus E1A, the SV40 large T antigen, and HPV16-E6/E7, but their effectiveness as research models has been hampered by both genetic instability, as well as a lack of normal growth and differentiation. This is likely because expression of oncogenes perturbs cell differentiation programs (Weinberg, 1998). For example, cell lines immortalized with SV40 large T antigen were found to stratify and make proteins that distinguish differentiated corneal epithelia, but they did not synthesize glycosylated MAMs (Gipson et al., 2003).

Newer cell lines utilizing an active version of the TERT gene for immortalization, have been more successful. TERT encodes the catalytic subunit of telomerase, an enzyme that repairs telomeres damaged during chromosome replication. Located at the ends of chromosomes, telomeres have been compared to the metal clips at the ends of shoelaces, the “caps” that prevent the shoelace from unraveling. A natural constraint on the ability to completely replicate chromosome ends leads to a shortening of telomeres, with each cell replication. At some point telomeres change from a “capped” state to an “uncapped” state, signaling cell senescence. Telomerase is active in development, but is silenced in almost all organ systems from the embryo onwards, except germ cells (and stem cells, to some extent). Adding an

active copy of TERT compensates for erosion of chromosome ends during the process of replication and makes the cell line carrying the gene functionally immortal.

Gipson and colleagues (Gipson et al., 2003) created a corneal epithelial cell line, HCLE, using a combination of strategies. Heeding reports that knockdown of CDKN2A (p16) and/or TP53 (p53) tumor suppressor pathways is necessary to immortalize human epithelial cells (Kiyono et al., 1998; Rheinwald et al., 2002; Weinberg, 1998), they first transduced primary cultures of human corneal–limbal and conjunctival epithelial cells with mutant CDK4 and dominant-negative TP53-expressing constructs. Then they added a TERT-expressing construct. When grown in high-calcium medium on plastic and type I collagen, cells of both lines stratified and differentiated. HCLE cells expressed corneal epithelial-specific keratins K3 and K12, and both HCLE and HCjE cells expressed K19. As in native tissue, both cell lines expressed MUC1, MUC4, and MUC16 and immunoreactive MUC1 and MUC16 proteins were localized to the apical cell layers of the stratified cultures. Importantly, both cell lines produced glycosylated mucins.

A second immortalized corneal epithelial cell line, hTCEpi, was developed from primary cultures of human corneal epithelial cells (Robertson et al., 2005). Heeding reports that CDKN2A induction can be bypassed under appropriate culture conditions, only a TERT-expressing construct was used. Indeed, it was observed that CDKN2A activity was gradually downregulated with increasing passaging of TERT-immortalized cells and did not require direct abrogation. Air-lifting produced a well stratified epithelium (five to seven cell layers) with apical ZO1-stained tight junctions. Submersed culture demonstrated increasing expression of stratification markers (keratins K5/K14) with K3-corneal keratin marker expression in long-term, air-lifted culture.

During limbal epithelial cell expansion *in vitro*, air-lifting has been shown to increase cellular stratification, enlarge surface cells, trigger cellular differentiation, and increase barrier function (Chen et al., 2017b). However, airlifting also appears to mimic some of the changes described in severe dry eye and squamous metaplasia, with reduced expression of mucosal markers (Li et al., 2008; Lin et al., 2014). A recent study examined mucosal marker expression in the hTCEpi epithelial equivalents created without airlifting (Yanez-Soto et al., 2015). Cells expressed MUC1, MUC4 and MUC16 mRNA and proteins, with a maximum between days 1 and 3 of the stratification process. Taken together, these studies suggest that airlifting should not be employed when mucosal differentiation of epithelial-equivalents is required.

At the same time as they created the HCLE corneal epithelial cell line, Gipson and colleagues developed a conjunctival cell line, HCjE using the same approach (Gipson et al., 2003). Other conjunctival cell lines have been created by other groups (Garcia-Posadas et al., 2017; Li et al., 2008; Lin et al., 2014). Conjunctival epithelial cell lines are especially useful, because there is not the tissue source that discarded donor corneal–limbal rims provide. They have been used as a model for dry eye and ocular surface inflammation.

1. Rose Bengal Exclusion—The most commonly used method for tracking damage to the ocular surface is staining with water soluble “vital” dyes (Abelson and Ingerman,

2005). This includes damage due to a variety of ocular surface diseases, including dry eye, (keratoconjunctivitis sicca), a desiccating condition of the ocular surface affecting 20% or more of the population in North America, Europe, and Asia (Craig et al., 2017). Vital dye staining is also observed after exposure to contact lenses soaked in certain multipurpose contact lens cleaning solutions (MPS), a phenomenon that has been called solution-induced corneal staining (SICS) (Maldonado-Codina et al., 2013). Similarly, the most frequently used preservative in topical eye drops, benzalkonium chloride, causes damage to the ocular surface and vital dye staining (Baudouin et al., 2010).

Fluorescein dye was first used clinically in 1882 for evaluation of corneal epithelial defects (Pflüger, 1882). Rose bengal dye became popular in the 1930s for dry eye diagnosis because of the distinctive “punctate” staining pattern observed at the ocular surface of patients (Sjögren, 1933). Rose bengal is now infrequently used in clinical practice, because of patient discomfort (Bron et al., 2015), but fluorescein continues to be used, and is the standard endpoint for clinical trials of investigational new drugs for dry eye (e.g., (Holland et al., 2017)).

Studies published in the early 1990s reported that healthy, living cells in monolayer culture, but not dead cells, take up rose bengal (Feenstra and Tseng, 1992b) and that uptake is blocked by addition of tear components such as mucins. Fluorescein is the parent compound from which rose bengal was derived; thus, the two dyes are closely related but differ somewhat in uptake properties (Kim, 2000). Living corneal epithelial cells in monolayer culture take up fluorescein in the same way as rose bengal, but at a lower level, requiring visualization under epifluorescent illumination (Feenstra and Tseng, 1992a). Unlike rose bengal staining, fluorescein uptake did not appear to be blocked by mucins added to monolayer cell cultures (Feenstra and Tseng, 1992a).

Later it was shown that human corneal epithelial cells in culture exclude rose bengal autonomously if they differentiate and elaborate a mucosal glycocalyx, i.e., develop into an epithelial tissue-equivalent with mucosal differentiation (Argueso et al., 2006). Inhibition of O-glycosylation by knockdown of T-synthase, a galactosyltransferase required for synthesis of core1 O-glycans, decreased surface O-glycosylation and increased dye penetrance. This demonstrated the importance of the O-glycans in forming a transcellular barrier to dye entry (Argueso et al., 2009). This also was the first indication that monolayer cell cultures, because they do not elaborate a mucosal glycocalyx, are not a valid model of the ocular surface. In contrast, the mucosal glycocalyx associated with the cell surface does not appear to be a significant barrier to fluorescein penetrance (unpublished observations).

Molecular knockdown experiments using the HCLE tissue-equivalent model have shown that MUC16 is essential for the exclusion of rose bengal dye (Blalock et al., 2007). MUC16 appears to be the major MAM component in promoting transcellular barrier to vital dye penetration. In fact, knockdown of MUC1 *decreased* dye penetrance (Gipson et al., 2014). The lectin LGALS3 (galectin-3), is required to cooperate in the exclusion of rose bengal, as inhibition of LGALS3 binding to MAMs resulted in increased rose bengal staining (Argueso et al., 2009). LGALS3 contains a conserved carbohydrate-binding domain with

affinity towards beta-galactosides and forms multimers via intermolecular interactions via its N-terminal domain. It can therefore mediate crosslinking of glycoproteins such as MAMs.

Thus, the transcellular barrier to dye uptake is comprised of extended, heavily glycosylated MAM EDs, pulled together into an organized lattice-like structure via LGALS3. The dominance of MUC16 over MUC1 may be due, not only to the exceptional length of its ED, but also its the heavy O-glycosylation, providing a substantial hydrophilic surface. MUC16 would provide more surface for glycan-galectin interactions to hold the molecules in a tight conformation. Abrogation of MUC1 with its shorter ED, would mean a more uniform glycocalyx, potentially resulting in a more substantial barrier. When the MAM repertoire is mixed, several levels of MAM-galectin association may be present with MUC16 EDs extending further from the cell membrane than MUC1 EDs. This uneven, mixed-length lattice could create spaces for dyes to reach the cell surface (Gipson et al., 2014).

We have been making use of HCLE corneal epithelial-equivalents with mucosal differentiation to investigate mechanisms of rose bengal staining due to damaging stress. Figure 8 shows the effect of oxidative stress on rose bengal dye uptake in the HCLE corneal epithelial-equivalents with mucosal differentiation. This effect is highly reproducible and quantifiable. One of our groups showed that benzalkonium chloride or MPS treatment of HCLE epithelial-equivalents with mucosal differentiation causes an increase in rose bengal staining, and we provided evidence for the associated shedding of a portion of the MUC16 large subunit (Gordon et al., 2011). More recently, we showed that oxidative stress causes shedding of LGALS3 into the culture medium (Webster et al., 2018). These results suggest that rose bengal staining of the ocular surface under conditions of stress is caused by disruption of the mucosal glycocalyx.

2. Desquamation—In the Introduction to this article, we discussed how the ocular surface epithelia are continually renewed in a process whereby daughter cells generated by division of basal cells at the basement membrane are displaced upward in the cell layers, become increasingly flattened and undergoing mucosal differentiation. Once differentiated, the apical layer of mucosal epithelial cells do not remain static, but are shed fairly rapidly, in a process called desquamation.

Scanning electron microscopy (SEM) of the ocular surface of various mammals has revealed a contiguous mosaic of polygonal cell shapes with a range of sizes, each having a light, medium, or dark appearance, the dark reflex cells being predominantly the largest (Doughty, 2016; Pfister, 1973). The dark reflex is due to a reduction in the size and number of microplacae and microvillae (Hazlett et al., 1980; Pfister, 1973). Within a short time, the most mature cells are shed from the cell surface and are replaced by the cells in the epithelial cell layer beneath. It has been estimated that new basal epithelial cells move upwards and are lost from the surface in a period of 3.5 to 7 days in mouse, rat, guinea pig, and dog (Hanna and O'Brien, 1960). Turnover time of the human corneal epithelium has been estimated to be on the order of 1 week (Hanna et al., 1961).

The molecular mechanisms controlling desquamation at the ocular surface are essentially unknown. Much more is known about the process in the epidermis ([Milstone, 2004](#)),

however, with its complex, stratified architecture, the epidermis may not be the best comparison. In the granular layer, epidermal keratinocytes undergo the last steps of their terminal differentiation program resulting in cornification. The coordinated conversion of living keratinocytes into corneocytes, the building blocks of the cornified layer, represents a unique form of programmed cell death (Eckhart et al., 2013). Keratinocytes activate anti-pyoptosis pathways to prevent inflammation and premature cell death during terminal differentiation (Eckhart and Tschachler, 2018).

In contrast to corneocytes, essentially all cells in the apical layer of the ocular surface epithelia remain alive, as evidenced by uptake of calcein dye (an indicator of cell viability) (Ren and Wilson, 1996). Some earlier references in the literature suggest that cell death during desquamation occurs by apoptosis, pointing to observations of a small percentage of ANXA5-binding or TUNEL-positive cells at the apical layer of human corneal epithelial tissue-equivalent culture (Jester et al., 2003), and at the apical layer of the normal corneal epithelium of mice (Strong et al., 2005; Yeh et al., 2003). However, other studies reported that the desquamation process at the ocular surface did not involve bleb formation or nuclear DNA laddering characteristic of apoptosis (Lomako et al., 2005; Ren and Wilson, 1996). The number of apoptotic cells increases considerably when the ocular surface is under desiccating stress (e.g., (Yeh et al., 2003{Bauskar, 2015 #1494}), suggesting that apoptosis is primarily a mode of cell death following damage.

Thus, a specialized form of cell death may be utilized at the ocular surface, as in the epidermis. In the adult rabbit, apical cells display ring-shaped depressions (“craters”) or full-thickness holes. The microvilli of the underlying cell can be seen at the base of a hole, having parameters consistent with a light cell (Pfister, 1973). The ring-shaped features are smallest on light cells, slightly larger on medium cells, and largest on dark cells (Doughty, 2006). These findings have suggested an orderly sequence of events leading to desquamation, which starts when a cell reaches the ocular surface. At that time, a hole forms and then expands in a controlled manner as the cell matures from a light cell into a dark cell. Hole formation exposes the underlying, less mature light cell. As the overlying mature cell is shed, the underlying light cell reaches the surface and the sequence of events begins again. Because hole formation exposes the well-formed microvillae on the underlying cells, it may represent a unique process for cell turnover that functions to maintain tear film stability (Pfister, 1973). Once shed, desquamated corneal epithelial cells no longer take up calcein; instead their nuclei stain with ethidium (an indicator of non-viability) (Ren and Wilson, 1996).

Corneocytes in the epidermis and epithelial cells in the apical layer at the ocular surface must actively dissociate themselves from neighboring cells to desquamate. In the epidermis, a critical event in this process includes the rearrangement of junctional desmosome complexes and their ultimate enzymatic digestion, regulated by the interplay between multiple proteases and their inhibitors (Has, 2018). In *Xenopus* frogs, ocular surface desquamation follows a circadian cycle and the proteinase MMP2 is located in the right place, at the right time, to disrupt intracellular junctional proteins (Wiechmann et al., 2014). Whether MMP2 is functionally involved in the process of desquamation remains to be determined.

A surface view of a human cornea, immunostained with an antibody to MUC1 or MUC16, reveals a mosaic of light, medium, and dark cells very similar to the ocular surface imaged by SEM (Gipson, 2014). The intensity of immunostaining correlates indirectly to the cell surface area, the largest (and presumably most mature) dark cells staining the least. Thus, as apical cells mature, membrane folds are reduced and cell area increases, MUC1/ and MUC16 are corresponding lost from the cell surface.

Two studies have implicated MAMs in desquamation of the ocular surface epithelia. The first employed a rat corneal epithelial-equivalent model with mucosal differentiation. Cells that appeared to be desquamating in culture exhibited a high level of Muc4 accumulation. Since Muc4 has been shown to be a potent anti-adhesive and a repressor of apoptosis in cancer cells, it was proposed that it might play a role in the non-apoptotic desquamation process in normal cells (Lomako et al., 2005).

A study using the HCLE tissue-equivalent model with mucosal differentiation implicated MUC16 in desquamation. Knockdown of MUC16 decreases transepithelial resistance, a measure of paracellular barrier integrity (Gipson et al., 2014). This was associated with decreased expression of *ZO1* and *OCLN*, which encode components of tight junctions. Knockdown of MUC16 also resulted in disruption of the actin cytoskeleton associated with tight junctions and reduced surface microvillae leading to greater apical surface cell area (Gipson et al., 2014). It is suggested that the ezrin binding domain in the ICD of MUC16 may mediate this effect, by interaction with the actin cytoskeleton of the microvillae. Loss of microvillae and the ‘lubricating’ membrane-associated mucin MUC16 may cause the cells to stick to secreted mucins in the tear film and facilitate their removal during desquamation (Gipson, 2004).

It will be important to follow up on these findings to define the process of desquamation at the ocular surface.

3. Barrier to Infection—MUC1 provides a substantial barrier to infection in various mucosal organ systems (reviewed in (Dhar and McAuley, 2019)). Two mechanisms for this protection have been described.

First, MUC1 serves as an adhesion receptor for various pathogenic bacteria and viruses (e.g. (Boll et al., 2017; Dhar et al., 2017; Lillehoj et al., 2015; Lillehoj et al., 2002; Linden et al., 2009; McAuley et al., 2017)). The MUC1 ED contains the glycan antigens Lewis^b, sialyl Lewis^a, and sialyl Lewis^x, which can serve as binding sites for the bacterial adhesins (Linden et al., 2009). MUC1 binds the respiratory pathogen *P. aeruginosa* via bacterial flagellin (Lillehoj et al., 2002). Rather than a way for pathogens to access cells however, binding to MUC1 appears to inhibit infection by triggering large subunit shedding (Lillehoj et al., 2015; Linden et al., 2009). In this way, the MUC1 large subunit serves as a releasable decoy, promoting bacterial and viral clearance.

MUC1 expressed on the surface of macrophages also binds pathogens. However, in this case, binding inhibits infection in a different way. Phagocytosis of *P. aeruginosa* by alveolar macrophages contributes to its clearance from the lungs (Dhar et al., 2017). Binding to

MUC1 on the surface of macrophages appears to be the first step in phagocytosis, as MUC1-deficient macrophages are inefficient at phagocytosing pneumococci.

If MUC1 binding of pathogens limits infection, then increasing the amount of MUC1 on the cell surface should be beneficial. *P. aeruginosa* stimulates alveolar macrophages to release TNFA, which induces MUC1 protein levels in airway epithelial cells (Dhar et al., 2017). Overexpression of MUC1 by epithelial cells or the addition of sialylated synthetic MUC1 constructs, reduced Influenza A viral infection *in vitro*. Respiratory Syncytial Virus and human metapneumovirus also stimulate MUC1 expression, consistent with a role in protection against infection (Banos-Lara Mdel et al., 2015; Li et al., 2010).

Respiratory Syncytial Virus is also a strong inducer of *MUC21* and *MUC22* expression (Banos-Lara Mdel et al., 2015). *MUC21* is one of the most upregulated RNAs in bronchoalveolar lavage fluid of children with pneumonia due to severe infections of the atypical bacteria *Mycoplasma pneumoniae* (Wang et al., 2017). These findings suggest that MUC21 and MUC22 are also involved in defense against infection.

Staphylococcus aureus is among the most common bacterial pathogens involved in ocular surface infection. *S. aureus* adheres to the surface of many cell types via the bacterial-encoded fibronectin-binding protein, and this also appears to be the case for corneal epithelial cells (Jett and Gilmore, 2002). The current model for fibronectin-binding protein-mediated adhesion and invasion proposes a fibronectin-dependent bridging between *S. aureus* fibronectin-binding proteins and host cell $\alpha_5\beta_1$ integrin (Massey et al., 2001). However, this model was developed from the findings of studies performed in monolayer cultures; other factors may be at play in mucosal epithelia. In fact, *S. aureus* does not adhere to apical cell surfaces in the presence of an intact glycocalyx (Govindarajan et al., 2012; Jett and Gilmore, 2002; Spurr-Michaud et al., 1988).

In the HCLE tissue-equivalent with mucosal differentiation, knockdown of MUC16 increased cell surface adherence of *Staphylococcus aureus* (Blalock et al., 2007; Gipson et al., 2014), but knockdown of MUC1 has the opposite effect (Gipson et al., 2014). This suggested to the authors that the barrier to pathogens is improved in the absence of MUC1, much like the barrier to rose bengal dye. Alternatively, MUC1 may be essential for bacterial adherence, but MUC16 interferes with bacterial access to MUC1. More work is needed to understand these alternatives better.

4. Dampening of the Innate Immune Response—Pathogens that penetrate the first line-of-defense at the glycocalyx are recognized by Toll-like receptors, a family of innate immune receptors expressed by epithelial cells and resident leukocytes. Toll-like receptors sense danger signals and pathogen-associated molecular patterns intrinsic to microorganisms and initiate an innate immune response (Basu and Fenton, 2004). For example, TLR5 recognizes bacterial flagellin, the major protein constituent of the flagella. This leads to NF-kappaB activation and induced expression and secretion of proinflammatory cytokines such as TNFA and IL8, thus stimulating inflammation.

Recent studies using primary human bronchial epithelial cells in culture have demonstrated that MUC1 coordinates with Toll-like receptors to control the resolution of acute inflammation essential to the prevention of chronic inflammatory disease. Thus, TGFA also activates EGFR, resulting in phosphorylation of the MUC1 CT. The activated MUC1 CT then associates with TLR3 and TLR5, inhibiting recruitment of TRIF and MYD88, suppressing NF-kappaB activation and thereby dampening the innate immune response (Kato et al., 2016). MUC1 also suppresses NF-kappaB activation in response to TLR3, 4, 7, and 9 agonists, suggesting that it may be a universal regulator of TLR signaling (Ueno et al., 2008).

This finding was recently confirmed and extended in the HCLE tissue-equivalent model with mucosal differentiation (Menon et al., 2015). It was found that knockdown of either MUC1 or MUC16 released dampened expression of the proinflammatory cytokines TNFA, IL6 and IL8 in response to ligand-activated TLR2 and TLR5 (activated with heat-killed *Listeria monocytogenes* and flagellin, respectively).

B. Mouse as a Model System

The mouse has become the premier mammalian model for disease research because of its small size, ease of genetic manipulation and relatively short generation time in comparison to other mammals (Justice and Dhillon, 2016). While humans and mice branched from a common ancestor approximately 80 million years ago, there are close physiological similarities between the two species. Nevertheless, humans and mice have adapted to different environments and so they have also evolved many differences in how they use molecules and molecular pathways that may not be as easily apparent (Perlman, 2016).

For many years, the rabbit was the preferred animal model for ocular surface research (Prince, 1964). This has changed; the mouse is now the “go to” model for investigation of ocular surface biology and disease mechanisms, including epithelial repair (Fini and Stramer, 2005; Saika et al., 2002) and dry eye (Barabino and Dana, 2004; Schrader et al., 2008; Stern and Pflugfelder, 2017), and for efficacy studies on investigational therapeutics (e.g., (Bauskar et al., 2015)). With regard to the biological role of MAMs, transgenic mice provide an important complement to cell culture models, as they enable a more comprehensive understanding in the full context of the organism. However, as we detail in this Subsection, there are molecular differences between the mucosal glycocalyx of humans and mice that must be taken into consideration. We believe this is the first time these differences have been comprehensively compared.

1. Ocular Surface System—The ocular surface system includes the corneal and conjunctival epithelia, the lacrimal glands, and the innervation connecting them. This has been called the “lacrimal functional unit” (Stern et al., 1998). The ocular surface system also includes the tear film, the eyelids, the Meibomian glands and accessory glands, the nasolacrimal duct and the integrative functions of the endocrine, immune, and vascular systems (Gipson, 2007).

The anatomy and physiology of the human and mouse ocular surface system is very similar, but not identical; the implications for function must be taken into consideration when using

mouse as a model. Figure 9 is a schematic of the mouse eye and ocular surface system. The size and position of the glands with respect to the eye are approximately to scale.

The mucosal ocular surface is kept continually wet by the tear film. Measurement of the tear film electrical profile in mice produced an average thickness of 7 μm (Tran et al., 2003). This is at the upper end of the range of measurements in humans (King-Smith et al., 2000; Wang et al., 2003). The tear film is composed of water, glucose, salts, and lipids, and proteins (including mucins). A small number of highly abundant proteins are estimated to comprise more than 90% of the total human tear protein by weight, including LYZ (lysozyme), LTF (lactoferrin), LCN1 (tear lipocalin) and LACRT (lacritin) (Zhou and Beuerman, 2012). The remaining 10% is highly complex; in the most comprehensive mass spectrometry list, 1543 tear proteins were identified (Zhou et al., 2012). At $\sim 30 \text{ ug/mL}$, abundance of the molecular chaperone CLU is substantially lower than that of the major tear proteins (e.g., ~ 50 fold less than LCN1 and ~ 10 -fold less than LACRT), but near the upper end of abundance for the other proteins. A recent proteomics analysis of mouse tears identified 139 different proteins (Karn and Laukaitis, 2015). Members of three large protein families were identified that have no counterparts in humans: androgen-binding proteins, exocrine secreted peptides and major urinary proteins. The last group are members of the lipocalin family that mediate female recognition of potential mates. Not surprisingly, CLU is found in mouse tears as in human tears, with a concentration estimated at $\sim 5 \text{ ug/mL}$ (Bauskar et al., 2015).

Plasma membrane ridge-like folds or pillar like projections, called **microplcae or microvillae**, project into the tear film from the surface of the apical epithelial cells at the ocular surface in all vertebrate species examined (around 150 nm high in guinea pig) (Nichols et al., 1983). Viewed coronally, these projections form different patterns in different species (e.g., (Doughty, 1990, 2004, 2016; Pfister, 1973)). The pattern of microplcae projecting from the surface of apical epithelial cells of the mouse ocular surface appears very similar to human (Danjo et al., 2000). The microprojections increase cell surface area, enhancing the stability of the tear film. In turn, the tear film smooths the ocular surface, neutralizing negative optical effects (Johnson and Murphy, 2004). Various staining techniques reveal a well-developed **glycocalyx** in both human and mouse e.g., (Gipson, 2007; Wells and Hazlett, 1984).

The **corneal** and **conjunctival epithelia** in mouse and human have a similar stratified squamous structure. However, the corneal epithelium of mouse has an average of 13 cell layers, which is about twice the number found in human (Henriksson et al., 2009). This is a result of an increase in squamous cell layers. Other epithelial features, such as desmosomal junctions, hemidesmosomes, and basement membrane are similar to human. The epithelium contributes $\sim 30\%$ percent of the total corneal thickness in mouse, but only $\sim 10\%$ of the total corneal thickness in humans (Li et al., 1997). Mouse corneal epithelial cells do not express the keratin K3, while humans and other mammals express the K3/K12 keratin pair (Chaloin-Dufau et al., 1993).

Goblet cells residing in the conjunctiva are secretory cells comprising the primary source of soluble and gel-forming mucins (Gipson and Inatomi, 1998). In humans, the conjunctival

goblet cells secrete MUC5AC; in mice, Muc5b is also secreted at lower levels (Gupta et al., 2011). Dendritic cells of the conjunctiva are located in close proximity to goblet cells, and evidence suggests they modulate one another's function (Contreras-Ruiz and Masli, 2015).

The submucosal glands supporting the ocular surface epithelia in humans include the **lacrimal glands** located in the anterior and lateral region of the roof of the orbit, and the accessory lacrimal glands located in the fornix of the conjunctiva and at the edge of the upper tarsus (Gipson, 2004). The lacrimal glands are the main source of the aqueous and serous (protein) components of the tears, although they produce some mucins and lipids too. In mammals, there are two types: 1) a superior lacrimal gland, with multiple ducts that open in the lateral half of the upper conjunctival sac and 2) inferior lacrimal glands, with only one duct that opens into the lateral canthus. Humans have only the superior lacrimal gland composed of a larger orbital lobe and a smaller palpebral lobe. Rodents have only the inferior gland, which is divided into an intra-orbital and extra-orbital portion, the latter located below the ear next to the parotid glands.

Two additional accessory glands also contribute to the aqueous component of the tears in humans: the **Krause** and **Wolfring glands**, whose ducts open in the conjunctiva. These glands have a mixed population of both serous and mucus cell types (Seifert et al., 1994). They are not found in mice (Sakai, 1989).

Transcriptomic analysis of lacrimal gland revealed that the most highly expressed genes differ between humans and mice (Ozyildirim et al., 2005). In humans, cDNAs encoding LYZ, LCN1, LTF, LACRT, PRR4 and PROL1 were most abundant. However, the top five transcripts in mouse corresponded to major urinary protein family member Obp1a; androgen-binding protein family member C2c (now called Scgb2b20); a novel hypothetical protein that was named lacrein, which bears some similarities to LACRT, and may be the mouse counterpart; Sptl1, a key enzyme in sphingolipid metabolism; and a putative hydrolytic enzyme similar to Lipf, also involved in lipid metabolism. These differences mirror the differences in the respective tear proteomes of human and mouse.

Found in both humans and mice are the **Meibomian glands**, named in honor of Heinrich Meibom, who was the first to describe them in detail. They are sebaceous glands that produce the lipid portion of the tear film, helping to prevent evaporation. The Meibomian glands are located side by side in a row, aligned perpendicular to the edge of both upper and lower eyelids. They are cluster-shaped, with multiple acini that lead into a central duct. The release of their content is induced by blinking (Ross and Pawlina, 2015). Acylated omega-hydroxy fatty acids (OAHFA) are the major amphiphilic component of meibum in both humans and mice (Butovich et al., 2012).

Also found in both humans and mice are the **Zeiss** and **Moll's glands** located next to the eyelashes. The first are sebaceous glands, associated with the hair follicle. Moll's glands are a type of modified sweat gland found at the base of the eyelashes. Both contribute to the lipid portion of the tear (Ross and Pawlina, 2015).

The **nasolacrimal duct** is a channel located in the medial canthus of the eye, which communicates with the nasal cavity. Its function is to absorb the components of the tears,

in this way helping to regulate the tear level. The **eyelids** are important, not only for being the place where glands are lodged, but also for their ability to move in the blink, covering the entire surface of the eye when closed. They constitute an additional defense barrier and distribute the content of the tear film, as well as help to renew them during the blink (Murube, 2009). The tear film must remain continuous between blinks in order to fulfill its function. Mice have a much slower blink rate than humans, reflective of the fact that mouse tear stability is markedly greater (Duke-Elder and Gloster, 1968).

Most tetrapods, including rodents, have a “third eyelid” or **nictitating membrane** (from Latin *nictare*, to blink); however, this structure is vestigial in humans (Heralgi et al., 2017). The transparent or translucent nictitans can be drawn across the eye from the medial canthus. It protects the ocular surface against trauma and maintains moisture, while also allowing for vision. The third eyelid is also believed to help keep the surface of the eye moist by holding the tear film against the cornea better than the eyelids do by themselves. Loss of the third eyelid through trauma or in the treatment of neoplasia frequently results in chronic irritation of the cornea and remaining conjunctiva (Murube, 2009). Attached to the nictitans is the **Harderian gland**, which can secrete mucous, serous fluid or lipid. In some animals, the Harderian gland may produce up to 50% of the tear film (Chieffi et al., 1996). Its disposition varies depending on the animal group; in rodents it is found around the back of the eyeball (Sakai, 1989). Like the nictitans, the Harderian gland is absent in humans. The greater tear film stability in mice as compared to humans may be due in part to the additional lipid secreted by the Harderian gland.

Like other mucosal epithelia, the conjunctiva contains **conjunctiva-associated lymphoid tissue** (CALT) (Knop and Knop, 2000; Reinoso et al., 2012). The component cells are thought to be a key location for the generation of adaptive immune mechanisms of the ocular surface. Dendritic cells in the human conjunctiva are detected in organized follicles of CALT and diffusely distributed through the stroma along with intraepithelial lymphocytes (Gandhi et al., 2013; Reinoso et al., 2012). Postnatal development and ultrastructure of CALT in the mouse is similar to humans (Siebelmann et al., 2013). Topical stimulation with *C. trachomatis* or ovalbumin/cholera toxin B led to CALT generation exclusively in the nictitating membrane (Steven et al., 2008). Electron microscopy showed intraepithelial lymphocytes and follicles consisting of lymphocytes, dendritic cells, and macrophages.

2. Genes—With publication in 2002 of the mouse chromosome 16 draft DNA sequence, and a full sequence comparison of human chromosome 19 with related mouse sequences, it became possible to draw preliminary conclusions about similarity of the mouse and human genomes for the first time (Copeland et al., 2002). While chromosome number and size differ between human and mouse, their genomes appeared remarkably similar, not only in how the genes are organized on the chromosomes, but also at the level of individual genes and their DNA sequences. However, that once the human and mouse genomes were fully sequenced and published in 2008 and 2009, respectively, a more complete story emerged (Church et al., 2009). Despite the striking synteny between homologous DNA segments, one-fifth of mouse genes are new copies that have emerged in the last 90 million years of mouse evolution. This helps to explain many of the differences that distinguish human and mouse biology.

Table 5 compares all human epithelial MAM genes with the corresponding mouse ortholog. The mouse genes are in their expected location based on chromosomal synteny between the two species. The exon count for individual genes is well-conserved.

Muc20 and *Muc4* lie adjacent to one another in mice, as in humans. However, in the mouse genomic region syntenic with the human *MUC3*, *MUC13* and *MUC17* gene cluster, only a single gene *Muc3* has been fully established. This may be because there are several gaps in the current mouse genomic assembly that make annotation challenging. Several genes upstream of *Muc3*: *Gm31160* and *Gm40349*, have been annotated, and they may represent additional Muc family genes. The Genome Reference Consortium is tentatively planning to release an updated mouse assembly this year, and the mouse genome will be reannotated soon after that becomes publicly available (Dr. Eric Cox, NCBI, personal communication).

The gene cluster of *MUC21* and *MUC22* at human cytogenetic locus 6p21.33 may legitimately be more streamlined in mice. When we examined the NCBI Gene database for mouse orthologs, we identified a predicted gene with similarity to both *MUC21* and *MUC22*. This was located just downstream of *MUCL3*, and just upstream of *HCG22*, similar to human *MUC21* and *MUC22*. The structure of the ambiguous gene, with 3 exons, was similar to human *MUC21*, but the coding sequence length was more like *MUC22*. A BLAST analysis revealed coding sequence similarity across the entire length of *MUC21*, while the similarity to *MUC22* was localized to the conserved Epiglycanin_C domain.

According to the NCBI Gene database, 92 organisms have a *MUC21* ortholog, while only 30 organisms have a *MUC22* ortholog. In addition to mouse, other rodents, including shrew mouse, Ryukyu mouse, Alpine marmot, golden hamster and naked mole-rat, all have *Muc21*, but appear to be missing *Muc22*. This preliminary analysis suggests that human *MUC22*, likely the result of a tandem gene duplication during evolution, is conserved in numerous related organisms, but is absent in mouse and other rodents.

We communicated this to NCBI in an effort to resolve the gene identity. The annotation of the gene related to both human *MUC21* and *MUC22* was changed in the NCBI database to identify it as *Muc21* (Dr. Eric Cox, NCBI, personal communication). It should be noted that *MUC22* was not found in human by automated algorithms, but instead required application of focused analysis techniques (Hijikata et al., 2011). Thus, it is possible that a separate *Muc22* gene may still be discovered in the mouse genome.

3. Proteins—Table 6 compares the polypeptide length of all human MAM proteins with their corresponding mouse orthologs, using data mined from NCBI databases.

One of the immediately obvious differences between human and mouse orthologs is the substantially shorter protein backbone length of the long MAMs. The length differences are 0.6-fold, 0.5-fold, and 0.5-fold for *Muc16/MUC16*, *Muc4/MUC4* and *Muc3/MUC3*, respectively, due to contraction of the VNTR. By contrast, the small MAMs show conservation of protein backbone length between mouse and human. This suggests that there is selective evolutionary pressure to maintain the VNTR length below a certain threshold in mice, and that there is pressure to exceed that threshold in humans.

The longer MUC backbones of humans contain more sites for O-linked glycosylation. Thus, it might be hypothesized that a longer backbone is more effective in maintaining ocular surface moisture and could be important in humans, which lack the third eyelid and associated Harderian gland present in most other mammals.

To test this hypothesis, we examined results from a classic study characterized the nictitating membrane in 22 species of subhuman primates (Arao and Perkins, 1968). Subhuman primates are transitional in evolution with respect to the third eyelid and Harderian gland. Table 7 lists the findings on nictitans coverage of the ocular surface from this study, comparing to the MUC16 protein backbone length, as mined from the NCBI database. The species covered by the two datasets did not match perfectly by species, but genus comparisons are included.

The nictitans eye coverage was found to vary widely among the different primate species, ranging from vestigial (human and chimpanzee) to the entire eye (Potto). The eye coverage increased consistently with increasing evolutionary distance from humans. However, the relationship between evolutionary distance and MUC16 length was not so clear cut. Great Apes and Old World Monkeys all have very similar MUC16 lengths – the longest of the primate families at ~14,000 amino acids – and nictitans coverage ranges from vestigial to 1/5th of the eye surface. New World Monkeys, the next lower evolutionary group, have much shorter MUC16 lengths (~4,000 to 5,500) and more substantial eye coverage by the nictitans (~1/3rd). However, Treeshrews, Lemurs and Bushbabies had widely variable MUC16 lengths, despite nictitans coverage of ~half the eye. We conclude that the reasons for the large variability of MUC16 length across species cannot be due simply to nictitans membrane and Harderian gland differences.

4. Gene Expression, Protein Localization—The three ocular surface MAMs documented in humans and mice localize differently at the ocular surface. Each is found primarily at the apical aspect of the ocular surface in both species. However, their distribution between corneal epithelium and conjunctival epithelia differs substantially. Figure 10 diagrams the distribution of these three MAMs at the ocular surface of human and mouse.

Unlike in humans, where MUC1 localizes uniformly across the surface of the corneal and conjunctival epithelia, very little Muc1 mRNA was detected in the corneal epithelium of mouse (Kardon et al., 1999). However, Muc1 mRNA was easily detectable in the mouse conjunctival epithelium, as well as the epithelium of the Harderian gland. The amount of Muc1 mRNA was about 5.5 times greater in the Harderian gland when compared to the conjunctiva. Similarly, an antibody to the Muc1 CT gave only a weak reaction in corneal epithelium of mouse, but reacted strongly with conjunctival epithelium (Kardon et al., 1999). The strongest staining was seen at the luminal aspects of the Harderian gland epithelium.

The stratified epithelia of the human conjunctiva and peripheral cornea express MUC4, but the amount of the protein attenuates with progression towards the central cornea (Inatomi et al., 1996). In mouse, mRNA extracted from cornea or conjunctiva was positive for Muc4

(Danjo et al., 2000; Lange et al., 2003). Likewise, Muc4 expression in the central and peripheral cornea, as well as the conjunctiva, was demonstrated by in situ hybridization. Muc4 antibody bound in a linear pattern along apical cells throughout the corneal and conjunctival epithelium (Lange et al., 2003). In rat, a similar localization is found in the ocular surface epithelia (Price-Schiavi et al., 1998; Swan et al., 2002).

The biggest difference between mice and humans is seen for MUC16. In humans, MUC16 is distributed uniformly across corneal and conjunctival epithelia, and in goblet cells. In mice, Muc16 has been identified only at the apical aspect of the conjunctival epithelium (Shirai et al., 2014) and on goblet cells (Wang et al., 2008). In contrast, the corneal epithelium appears to lack MUC16.

Thus, Muc4, appears to substitute for Muc16 at the surface of the mouse cornea. This means the MAM length at the corneal epithelial surface is effectively about 4-fold shorter in mice than in humans.

In order to make full use of the mouse model for functional studies, it will be important to extend the characterization of MAM gene expression to Muc21.

5. Knockout Mouse Findings—To date, knockout mice lacking the MAMs *Muc1*, *Muc4*, *Muc13* and *Muc16* have been generated. Homozygous lines are viable and fertile, with no apparent anatomical defects. Reviews have been published describing the effects of *Muc1* and *Muc16* deficiency in cancers and gastrointestinal tract inflammation (Joshi et al., 2015), and at the ocular surface (Shirai and Saika, 2015). Reported phenotypes are listed in Table 8. This list is not comprehensive for *Muc1*.

An examination of results listed in Table 8 leads to the general conclusion that loss of a specific MAM, no matter which one, leads to common phenotypes: inhibition of cancer growth and progression in the case of cancer models, promotion of infection and inflammation in infection models, and increased inflammation and fibrosis in the unperturbed state or in injury models. Perplexingly however, just the opposite is observed in a small number of these studies. These anomalies are underlined in blue in Table 8. Four different explanations have been offered.

The first explanation is raised in findings using the *Muc1* knockout mouse in cancer models. *Muc1* is overexpressed and aberrantly glycosylated in adenocarcinomas and in hematological malignancies. Epithelial tumors in *Muc1* knockout mice exhibit reduced growth when compared to congenic controls (Besmer et al., 2011). This is consistent with other studies supporting a role for *MUC1* as an oncogene promoting tumor development, progression, metastasis and resistance to chemotherapeutics. However, a surprising lung tumor-suppressing effect was observed when the *Muc1* knockout mouse was bred into the NNK A/J mouse lung cancer model (Xu et al., 2017). In studies to address mechanism, human MUC1 was found to suppress EREG production in both normal fibroblasts and malignant cells. The authors favor an explanation in which Muc1 exhibits distinct functions in epithelial, stromal and cancer cells in the tumor microenvironment, and that the sum of these effects results in tumor suppression.

A second reason for anomalous results may be due to compensatory mechanisms, whereby loss of one mucin is compensated by changes in expression of another. This phenomenon may mask the significance of a given MAM in tissue homeostasis when studied using knockout mouse models. For example, homozygous *Muc1* knockout mice exhibit a compensatory increase in *Muc4* in mammary tissue (Spicer et al., 1995), while homozygous *Muc16* knockout mice demonstrate reduced *Muc1* expression in the uterus and lung (Cheon et al., 2009). Knockout of multiple MAMs in the same mouse could potentially circumvent such compensatory mechanisms and provide further insights into their regulatory interplay.

A third factor that complicates analysis occurs when myeloid-derived immune cells contribute to the process under observation, since these cells also express *Muc1*. Studies show that loss of *Muc1* can affect the immune cell repertoire that develops from the bone marrow (Poh et al., 2009). Thus, phenotypes in *Muc1* knockout mice could be due either to loss of *Muc1* locally from the organ being examined, or loss of *Muc1* from infiltrating immune cells. The relative role of *Muc1* expressed locally in a tissue versus infiltrating immune cells can be examined experimentally by creating chimeric mice. This is done by transplanting a knockout or normal mouse with normal or knockout bone marrow, respectively. Studies in two gastrointestinal infection models that used the chimeric mouse approach confirmed the importance of making this assessment (McAuley et al., 2007; McGuckin et al., 2007; Ng et al., 2016; Ng and Sutton, 2016).

The last factor is mouse genetic background, as differences in the immune response among different inbred mouse strains are well-documented (Sellers, 2017). Results of two different studies suggest that genetic background may be a factor affecting the ocular surface phenotype of *Muc1* knockout mice. In the first study, *Muc1* knockout mice were found to be predisposed to developing eye inflammation when compared to normal littermates (Kardon et al., 1999). However, a different lab found no differences between *Muc1* knockout mice and littermate controls (Danjo et al., 2000). Ocular surface epithelia of knockout mice had a normal appearance of surface microvillae, a well-developed glycocalyx on the apical cell membrane, and a normal appearance of goblet cell mucin packets. There was no convincing evidence that bacterial adherence on the cornea was increased. *Muc4* expression was not upregulated in *Muc1* knockout mice compared with control. No ocular surface infections were observed in *Muc1* knockout mice, which were housed in the animal facility over a period of 26 months.

There is only one other published study on the ocular surface phenotype of MAM knockout mice, and this utilized the *Muc16* knockout. A careful examination of the ocular surface revealed no gross defects (Shirai et al., 2014). However, tissue analysis revealed basal-like cells in the suprabasal layer of the corneal epithelium, with an increase in cell proliferation. The loss of *Muc16* accelerated regeneration of an experimentally-created corneal epithelial defect. In the conjunctiva, transcription factors STAT3 and JUNB were activated, and the cytokine IL6 was upregulated. The incidence of myofibroblast appearance and macrophage invasion were more marked in knockout stroma than in wild-type stroma after epithelial repair. Thus, the loss of *Muc16*, which is expressed only in the conjunctiva, still affects homeostasis of the corneal epithelium and stroma.

C. Insight from Human Genetics

While we cannot experimentally manipulate the genome of humans as we do in mice or cultured cells, we can study the effects of naturally-occurring mutations and polymorphisms to obtain insight into function of the associated gene. As in mouse transgenic and knockout models, human genetic findings are hypothesis-generating. For the most part, these hypotheses still need to be addressed, and thus provide a good way to conclude this article. In this section, we discuss evidence for function of MAMs expressed at the ocular surface from two different types of inherited disorders: 1) monogenic diseases and 2) complex diseases.

Monogenic diseases are caused by alterations in a single gene, and they segregate in families according to classic Mendelian principles of inheritance. As a rule, genetic diseases with Mendelian inheritance patterns are caused by mutations in the coding region of the gene. Dominant inheritance patterns manifest a disease phenotype when only a single copy of the mutant gene is inherited. So-called “gain-of-function” mutations usually result in toxicity of the mutant protein, providing little biological insight. On the other hand, “dominant-negative” mutations, which interfere with the function of the protein encoded by the wild-type gene copy, may give insight into biological role.

Recessive inheritance patterns require that both copies of the gene are mutated for the disease to manifest, due to “loss-of-function”. Recessive mutations are much like knockdown in cell culture or in mouse knockout models, often being informative of the gene’s biological role. Dominantly inherited phenotypes resulting from a reduction in functional gene dosage may similarly provide functional clues.

The vast majority of human diseases with a genetic contribution are multifactorial, also referred to as complex diseases. Examples include cardiovascular disease, cancer, diabetes, psychiatric disorders and glaucoma. Complex diseases are caused by variation in many genes. The variants can be single nucleotide polymorphisms (SNPs) or short deletions/insertions, duplications, and inversions. Each gene variant associated with a complex disease confers a degree of risk, but the presence of the variant does not necessarily mean the disease will be manifest.

Complex disease genetics is investigated through genome-wide association studies (GWAS). Genomic microarrays are used to analyze millions of variants at one time and investigate their statistical association to a disease phenotype or “quantitative trait”. Unlike genetic diseases with Mendelian inheritance patterns, about 88% of GWAS hits are intergenic or intronic (Hindorff et al., 2009). These are typically located in gene regulatory regions and control the expression level of the gene and its protein product.

It should be noted that, since microarrays do not contain every possible SNP (or other variant), significant SNPs discovered by GWAS usually are only a “tag” of the causative polymorphism. That having been said, current genomic microarrays sample SNPs very densely across the genome and analysis of SNPs not included in the microarray, located physically close to the tag SNP and in linkage disequilibrium (i.e., inherited along with the

tag SNP), can often identify likely causative SNP(s) and provide functional insight (e.g., (Jeong et al., 2015)).

1. MUC1 and Ion Channel Stability—As noted in Section III, the number of tandem repeats within the VNTR of a given MAM can vary considerably among individuals within a population (Gendler and Spicer, 1995). As an example, in a study examining the *MUC1* gene of 69 northern Europeans, the number of tandem repeat units varied from 21 to 125 (Gendler et al., 1990). In numerous studies, *MUC1* VNTR polymorphisms have been linked to susceptibility for both *H. pylori*-induced gastritis and gastric cancer. Individuals with short *MUC1* alleles are at a higher risk (Carvalho et al., 1997; Silva et al., 2001; Vinall et al., 2002). Homozygotes for small *MUC1* VNTR alleles were significantly associated with gastric carcinoma as well as with chronic atrophic gastritis and incomplete intestinal metaplasia, the two well-established precursor lesions of gastric carcinoma, suggesting that *MUC1* genotypes may define different susceptibility backgrounds in the gastric carcinogenesis pathway (Silva et al., 2001). At this time, we are not aware of any MAM VNTR polymorphisms associated with susceptibility to ocular surface disease, but it seems likely they will be discovered.

A monogenic disease caused by a VNTR mutation is medullary cystic kidney disease, an autosomal dominant kidney disorder leading to end stage renal disease. In a study employing DNA sequencing combined with other molecular techniques (Kirby et al., 2013), it was found that each of six affected families harbored an equivalent, but apparently independently arising, mutation in the *MUC1* gene: the insertion of a single C in one copy (but a different copy in each family) of the tandem repeat unit within the VNTR domain. In all cases, the insertion was predicted to cause a translational reading frame shift. This creates a new stop codon that terminates translation prior to large subunit cleavage site.

How the *MUC1* mutation causes disease pathology is not known, however, the cumulative effects of mild toxicity due to gain-of-function could explain some of the symptoms, e.g., fibrosis. As these patients rarely have cysts, the disease has been renamed autosomal dominant tubule-interstitial kidney disease due to *MUC1* mutations, abbreviated as Mucin 1 Kidney Disease (Al-Bataineh et al., 2017). Affected patients affected exhibit only renal disease, despite the presence of mutant *MUC1* protein in the epithelial cells of multiple organs, including at the ocular surface (Al-Bataineh et al., 2017). This points to the likely mild nature of mutant protein toxicity. It is important to remember that affected patients exhibit one wild-type copy of *MUC1* and this may be sufficient for function in most organs and sub-organs, including the ocular surface.

A clue to the natural role of *MUC1* in the kidney was provided by identification of another gene implicated in medullary cystic kidney disease, *UMOD*. The shed ED of *UMOD*, also known as Uromodulin or Tamm-Horsfall protein, is the most abundant protein in urine. Mice expressing *UMOD* with the disease-causing human mutations have less urinary *UMOD*, and also exhibit hypercalciuria and renal calcium crystals corresponding with reduced immunostaining for the renal calcium channel TRPV5 (Nie et al., 2016). In transiently transfected HEK293 cells, co-expression with *UMOD* or addition of exogenous *UMOD*

increased TRPV5 surface currents, reduced TRPV5 endocytosis and increased TRPV5 cell surface expression, consistent with a role of the shed UMOD ED in stabilization of TRPV5.

Because of similarities between the two proteins, similar studies were carried out with MUC1, with similar results. Interestingly, urinary MUC1 is also reduced in patients with calcium nephrolithiasis, a common type of kidney stone. Cell culture studies revealed that TRPV5 surface expression is also enhanced by binding LGALS3 (galectin-3). The MUC1 enhancement of TRPV5 surface expression proceeds by LGALS3-dependent crosslinking of O-glycans on MUC1 with the N-glycan on TRPV5.

Thus, LGALS3 crosslinking of MUC1 with the TRPV5 ion transport channel at the surface of epithelial cells appears to provide a novel mechanism for regulation of their function. It seems likely that the MUC1 ED might more broadly enhance surface expression of transient receptor potential (TRP) family ion transport channels by a similar mechanism of crosslinking and maintenance at the cell surface. For example, a large genome-wide association study focused on serum concentrations of cations revealed that the highest association with low serum magnesium levels (hypomagnesemia) was a very common genetic variant of *MUC1* (rs4072037) that adds nine amino acids to the extracellular N-terminus of the protein (Meyer et al., 2010).

A SNP in the magnesium transporter TRPM6 was also associated with low serum magnesium but to a lesser extent than the *MUC1* variant. Interestingly, the MUC1 SNP was associated with higher bone mineral density and lower fasting glucose levels which could proceed by a direct interaction of either the transmembrane MUC1 or shed MUC1 with transporters within the kidney tubule.

The findings in kidney suggest possible parallels to the ocular surface. TRP channels have been identified in the corneal epithelium (TRPV1, TRPV3, TRPV4, TRPM8, and TRPC4), in the conjunctiva (TRPV1, TRPV2, and TRPV4), and in the eyelid (TRPM8) (Reinach et al., 2015). These channels are expressed by the epithelial cells. TRPA1, TRPV1 and TRPM8 are also expressed on corneal afferent nerve endings of the ophthalmic branch of the trigeminal nerve (Reinach et al., 2015). TRPM8 is a cold-sensing receptor activated in ocular surface nerves after evaporation of the tear film (which results in cooling), thus regulating wetness of the ocular surface (Parra et al., 2010). Nerve TRPV1 is activated by hypertonic challenge, which in turn leads to an increased release of pro-inflammatory cytokines (Pan et al., 2011). Dysfunction of these ion channels has been suggested as a possible pathophysiological mechanism in dry eye disease (Belmonte et al., 2017). Whether MUC1 can regulate TRP channel activity at the ocular surface will be an interesting question to investigate.

MUC1 has also been linked to another ion channel, CFTR, the cystic fibrosis transmembrane conductance regulator. CFTR is an ABC transporter-class ion channel protein that plays a critical role in the transmembrane transport of chloride. It is the driving force of fluid transport in various epithelial cells. Mutations in CFTR cause cystic fibrosis, a disease with a recessive inheritance pattern – homozygosity for a specific CFTR mutation – or compound heterozygosity for two different mutations. The disease manifests as disruption of

exocrine function of the pancreas, intestinal glands, biliary tree, bronchial glands and sweat glands. Cystic fibrosis has long been established as a disease involving excessive mucus accumulation. A major symptom is the buildup of thick, sticky mucus in the lungs, which leads to life-threatening lung infections.

Surprisingly, *Cftr* knockout mice had lower levels of RNA expression and similar levels of protein for secreted mucins Muc2 and Muc5ac, as well as Muc3. However, there was a six-fold increase in Muc1 RNA expression in the colon of the *Cftr* KO mouse and a moderate increase in Muc1 protein. Breeding of the *Cftr* knockout mouse onto a *Muc1* null background, resulted in mice with a significant reduction in intestinal mucus accumulation (Parmley and Gendler, 1998). It was proposed that Muc1 predominantly contributes to mucinous obstruction of the gastrointestinal tract during cystic fibrosis.

The role of *Cftr* in lacrimal gland function has only recently received attention. Studies in rabbit demonstrated that *Cftr* is localized in both acinar and ductal cells, with its predominant presence in the ducts, suggesting it may play a key role in lacrimal ductal fluid secretion (Lu and Ding, 2012). Indeed, clinical studies have reported dry eye symptoms in cystic fibrosis patients (e.g., (Sheppard et al., 1989)). Moreover, a significant reduction in tear secretion is observed in *Cftr* KO mice, which also develop ocular surface disease, as evidenced by vital dye staining (Berczeli et al., 2018). It will be interesting to learn whether MAMs play a role in stabilization of the *Cftr* ion channel in the lacrimal gland.

2. MUC21 and MUC22—As noted in a prior section, *MUC21* and *MUC22* are located within the major histocompatibility complex (*MHC*) on chromosome 6. It encodes over 160 proteins of diverse function, at least half of which are directly involved in immune responses, including genes for major histocompatibility complexes HLA-A, HLA-B, and HLA-C. This is significant in view of the accumulating evidence for MAM roles in dampening of the immune response.

The *MHC* is the most polymorphic part of the human genome and *MUC22* exhibits more coding sequence alleles than even most *HLA class I or II* genes (Norman et al., 2017). Several polymorphisms link *MUC21* and *MUC22* to ocular surface diseases with an immune component.

A SNP in the intragenic region upstream of *MUC21* (rs2844682) (Yang et al., 2014) has been associated with predisposition to Stevens-Johnson syndrome, a hypersensitivity complex affecting skin and mucous membranes, which manifests at the ocular surface as severe, mucous-deficient dry eye. Mechanisms of this disease are poorly understood, however other genes associated with this disease suggest defects in pathways of adaptive and innate immune responses, sensing/processing of microbial and danger signals, and inflammation.

A SNP in *MUC22* (rs17190071) was identified associating with Behcet's disease, another genetically complex condition, characterized by recurrent inflammatory attacks affecting the orogenital mucosa, eyes and skin (Remmers et al., 2010). SNPs linked with systemic lupus erythematosus (Fernando et al., 2012; International Consortium for Systemic Lupus

Erythematosus et al., 2008) and psoriasis (Feng et al., 2009) were associated with both *MUC21* (rs886403, rs9295938) and *MUC22* (e.g. rs3871466, rs9366764, rs13191258); these autoimmune diseases also have ocular surface involvement.

MUC21 and *MUC22* have also been linked to respiratory disease. Polymorphisms in the *MUC21-MUC22* region were associated with asthma by admixture mapping and GWAS in the Latino population (Galanter et al., 2014). Polymorphisms in the *MUC22-HCG22* region were associated with Japanese late onset asthma (Yatagai et al., 2016) and childhood asthma in the Chinese population (Chen et al., 2017a).

Clues to *MUC22* function are provided by its association with diffuse panbronchiolitis (DPB), a rare, complex genetic disease of the respiratory system. DPB mainly occurs among the Japanese but has been reported in other (mostly east Asian) populations. A SNP in intron 2 of *MUC22* was positively associated with DPB (higher disease risk). In addition, a VNTR polymorphism in exon 3, which greatly reduces the size of the molecule (1,890-base pair deletion), was negatively associated with DPB (lower disease risk) (Hijikata et al., 2011).

DPB causes nodule-like lesions of respiratory bronchioles, chronic sinusitis, and intense coughing with large amounts of sputum production. The term *diffuse* signifies that lesions appear throughout both lungs, while *panbronchiolitis* refers to inflammation found in all layers of the respiratory bronchioles. Symptoms occur from the second to the fifth decade of life and are slowly progressive, ultimately resulting in respiratory failure if untreated.

Infection the bronchioles by bacteria such as *Haemophilus influenzae* or *Pseudomonas aeruginosa* can cause their infiltration by inflammatory cells; thus, treatment of DPB involves long-term use of macrolide antibiotics. However, when infection is resolved, inflammation often continues, for unknown reasons. Inflammation can be so severe that nodules containing inflammatory cells form in the walls of the bronchioles. Inflammation and infection also result in the production of excess mucus. The combination of inflammation, nodule development, infection, mucus, and frequent cough contributes to the breathing difficulties of patients.

The genetic study mentioned above (Hijikata et al., 2011) investigated *MUC22* expression in a tissue-equivalent model of primary human bronchial epithelial cells. Significantly, expression of *MUC22* mRNA was increased more than 100-fold by treatment with polyinosine-polycytidylic acid (double-stranded RNA) or lipopolysaccharide, which mimic viral or bacterial infection, respectively. Moreover, immunostaining with *MUC22* antibody was much more intense in the cytoplasm of serous cells of the lung submucosal gland from patients with DPB as compared to normal.

These intriguing results suggest two hypotheses about the pathogenesis of DPB and the role of *MUC22* in the normal lung. First, the SNP associated with disease in *MUC22*'s second intron might be a regulatory polymorphism that results in excessive *MUC22* production and large subunit shedding into the bronchioles. Second, *MUC22* could be directly involved in regulation of periciliary fluid secretion and mucous clearance in the bronchioles following infection.

Of interest with relation to our hypotheses, a connection to cystic fibrosis has also been considered in the search for a cause of DPB. Much like DPB, cystic fibrosis shows a genetic predominance among one geographic group to the rarity of others. Thus, while DPB dominates among East Asians, cystic fibrosis mainly affects individuals of European descent and is the most common genetic disease of this group (Elborn, 2016) A common polymorphism in this gene occurs in Asians not necessarily affected by either disease. Whether CFTR could contribute to DPB is still under investigation.

How might these findings relate to the ocular surface? Lacrimal glands secrete the serous component of the tears and are thus analogous to the serous cells of the lung's submucosal glands. We provide new data in Section II demonstrating that MUC22 is robustly expressed in the serous acini of the lacrimal gland.

Could genetic polymorphisms that cause DPB also cause lacrimal gland disease? Currently we have no information about this point (Naoto Keicho and Minako Hijikata, personal communication). DPB is managed by pulmonary physicians, to whom patients may not relate their eye symptoms. Considering the findings on MUC22 expression in the lacrimal gland, a possible relationship between DPB and lacrimal gland disease due to pathogen infection should be considered.

VI. Conclusions and Future Directions

MAMs are the defining molecules of the mucosal epithelial glycocalyx. A comprehensive structure/function characterization of MAMs at the ocular surface is extremely important to development of strategies for manipulating the glycocalyx to therapeutic advantage. While much progress has been made, much more is needed. In this article we update, including with previously unpublished data, the list of MAMs expressed by, and localized to, the ocular surface and lacrimal gland. They may now be recognized as MUC1, MUC4, MUC16, MUC21 and MUC22. In addition, MUC20 is expressed by the corneal/conjunctival epithelia, but is localized to deeper cellular layers. We then go on to update what is currently known about the structure/function of these MAMs, compiling known information with new, previously unpublished sequence analyses. The compiled information is then considered in relation to biological roles. Taken together, this information allows us to arrive at some conclusions about the MAMs expressed at the ocular surface as a group, and to generate hypotheses for the next stage of investigation.

As discussed herein, it is increasingly appreciated that MAMs play an important role as cell surface receptors that sense the extracellular environment and transduce signals intracellularly. It has been stated that the next big frontier in the MAM field is to expand our knowledge of their function in intracellular signaling (van Putten and Strijbis, 2017). With regard to the ocular surface, this frontier is vast. In cancer cells, MAM activation or inhibition of intracellular signaling cascades has been shown to regulate the biological processes of inflammation, cell-cell interactions, differentiation and apoptosis (Constantinou, 2011; Hollingsworth and Swanson, 2004; van Putten and Strijbis, 2017). But how activation or inhibition of intracellular signaling translates to roles at the ocular surface, with its non-proliferative, specialized squamous epithelial cells, is still largely unknown.

Our compilation of structure/function data and further analysis in Section III indicates that interaction with EGFR receptor tyrosine kinase family members is a common theme for all MAMs expressed at the ocular surface. A tyrosine residue in the MUC1 CT is phosphorylated by EGFR family members and it is predicted that a tyrosine residue in the MUC16 CT is also phosphorylated by EGFR family members. Moreover, MUC21 and MUC22 each has a tyrosine in their CT predicted to be phosphorylated by EGFR family members. Thus, even though the amino acid sequences of their CTs differ, it appears that these MAMs may converge functionally in this way.

EGFR phosphorylation of the MUC1 CT affects the binding to CTNNB1, which modulates the strength of the adherens junction connecting individual epithelial cells. This may be another common signaling theme among the MAMs. Like MUC1, the MUC16 CT binds to CTNNB1-CDH1 complexes and evidence suggests that this is through ezrin/radixin/moesin (ERM) actin-binding proteins, as discussed in Section IV. We also identified possible phosphorylation of some of the other MAM CTs by protein kinases that regulate intracellular adhesion. In cancer cells, changes in intracellular adhesion promote malignancy. In the apical epithelial cells of the ocular surface, the most logical link is to desquamation. As so little is known about desquamation at the ocular surface, this will be a significant area for future investigation.

As discussed in Section III of this article, MUC1 translocation has also been linked to the regulation of intracellular adhesion. These studies were made possible by the development of antibody reagents specific for the MUC1 CT. MUC16 also translocates to the nucleus, but nothing is known about the function. New, specific antibodies generated against the MUC16 CT have been recently reported (Aithal et al., 2018; Gipson et al., 2017). These will be invaluable for advancing this area of investigation. Similar reagents are needed for MUC21 and MUC22.

Besides EGF, the inflammatory cytokine TGFA can activate EGFR family receptors. As also discussed in Section IV, EGFR phosphorylation of the MUC1 CT initiates a signaling cascade that dampens the innate immune response, through association with toll-like receptors. This finding was confirmed in the ocular surface model and extended by demonstration that MUC16 also participates in this pathway. As noted above, the newer ocular surface MAMs, MUC21 and MUC22, have the potential for EGFR phosphorylation of the CT and genetic evidence presented in this article suggest that might also play a role in the innate immune response.

Many (if not most) ocular surface diseases are thought to have an autoimmune component (although the autoimmune antigen often has not been identified). For example, in dry eye, Inflammatory mediators and exposure of autoantigens at the ocular surface due to desiccating stress leads to an auto-immune-like adaptive T cell-mediated response. With amplification, this becomes a “vicious cycle of inflammation”, driving disease pathology (Pflugfelder and de Paiva, 2017). Individual TLRs are specific for different pathogens and danger signals (Moresco et al., 2011). Thus, the specific TLR with which a MAM interacts could provide for a unique functional response. This idea must still be addressed.

Indeed, results of expression studies discussed in Section IVC3 suggest that individual MAMs respond selectively to different pathogens. Our sequence analysis of the MAM CTs predicted sites for protein kinase A phosphorylation as a common theme. Protein kinase A lies downstream of a subgroup of G protein-coupled receptors (GPCRs) that couple with the heterotrimeric Galpha(s) protein. GPCRs respond to a diverse array of extracellular signals. Galpha(s) activates adenylyl cyclase, which elevates intracellular cAMP levels, activating protein kinase A. Elevation of cAMP is used by the cell in myriad ways to modulate innate immune functions (McDonough and Rodriguez, 2011); pathogens utilize mechanisms to reduce intracellular cAMP to suppress these functions (Serezani et al., 2008). Perhaps then, protein kinase A phosphorylation of MAM CTs is also involved in dampening the innate immune response. This idea, while entirely conjectural, can be approached experimentally.

MUC20 is something of an enigma. As presented in Section III, sequence analysis suggests that the *MUC20* gene arose from duplication of a portion of the *MUC4* VNTR domain, then underwent divergent evolution. It is the MAM mRNA most highly expressed in the apical layer of the human conjunctiva. However, the protein is found predominantly in intermediate cell layers of the corneal epithelium, and it is not detectable in tears, suggesting it plays a different role than the other ocular surface MAMs. MUC20 clearly associates with the plasma membrane, but does not appear to have a transmembrane domain. Nevertheless, recombinantly-expressed human MUC20 was shown to associate via its C-terminal domain with MET, a receptor tyrosine kinase activated by the extracellular ligand HGF. Interaction with MUC20 attenuated HGF-induced activities in kidney epithelial cells. In cornea, HGF is known to facilitate the migration and proliferation of epithelial cells, and to inhibit apoptosis. How this might relate to the role of MUC20 in ocular surface epithelia is, at this time, unknown. However, it may be speculated that MUC20 plays a role in regulating epithelial cell dynamics in the multi-layered epithelia.

When compared to the other MAMs localized to the ocular surface, MUC4 presents some intriguing differences. This MAM has the special capacity to directly activate EGFR family receptors adjacent to it in the plasma membrane via an EGF-like domain on its ED. However, it seems unlikely that activated EGFR family members phosphorylate the very short MUC4 CT, which has only a single tyrosine residue that is not predicted to be a phosphorylation site.

MUC4 appears to substitute for MUC16 in the corneal epithelium of mice and rats, but there are no obvious binding sites for proteins in the CT that might participate in regulation of the adherens junction. A difference between the CT of mouse/rat and human may be significant however; the mouse/rat CT is slightly longer than human, creating a predicted site for serine phosphorylation by casein kinase II. Intriguingly, casein kinase II phosphorylation of CDH1 increases its interaction with CTNBN1, strengthening the adherens junction and intracellular adhesion (Lickert et al., 2000). Perhaps MUC4 competition for casein kinase II binding regulates adherens junction strength, an idea that can be experimentally tested. However, while MUC16 levels decrease as cells age and knockdown studies suggest that this may lead to changes that precede desquamation, MUC4 levels are reported to be higher in desquamating cells. Thus, if these two MAMs substitute for one another in regulating desquamation, it seems likely that they do so via evolutionarily convergent mechanisms.

Encouragingly, analysis of *Muc16* knockout mice revealed effects on epithelial dynamics, as discussed in Section IV.B.5. Basal-like cells were observed in the suprabasal layer of the corneal epithelium, with an increase in cell proliferation. The loss of *Muc16* accelerated regeneration of an experimentally-created corneal epithelial defect. The recent availability of the *Muc4* knockout mouse now provides the opportunity for more significant results. We predict phenotypes related to epithelial cell dynamics, desquamation, rose bengal exclusion and dry eye disease.

Drugs used to treat ocular surface disease and glaucoma are applied topically to the ocular surface. Corneal and conjunctival epithelia are the key tissues in absorption of these drugs, however, the role of the mucosal barrier in drug delivery to the ocular surface is still essentially undefined (Ruponen and Urtili, 2015) and will be a very important area for future research. MAMs may decrease or increase ocular bioavailability depending on the magnitude of their role as barrier or retention sites, respectively. In every way examined to date (e.g., (Bauskar et al., 2015; Mauris et al., 2013; Pflugfelder et al., 2005)), ocular surface barrier function in mice is equivalent to humans, despite substitution of *Muc4* for *MUC16*, and despite the fact that the mouse *Muc4* ED is shorter than the ED of human *MUC4*. Thus, the mouse can provide an important model for studies of factors determining drug absorption at the ocular surface, and it will be important to fully characterize structure/function of the mucosal glycocalyx.

We end this Section with a final, very conjectural, but intriguing hypothesis about a new MAM mechanism. In Section V.A.1, we discuss genetic evidence for the role of *MUC1* in enhancing the functionality of a TRP family ion channel involved in calcium resorption in the kidney. *MUC1* stabilizes the ion channel at the surface of the cell, inhibiting its endocytosis. MAM stabilization of ion channels is a novel mechanism, not previously considered. It is hard to imagine that the TRP ion channel is the only example. As we discussed, TRP ion channels have been linked to ocular surface disease. In Section V.A.2, we conjecture that *MUC22* might similarly stabilize another type of ion channel, CFTR, thus regulating periciliary liquid secretion and mucous clearance in the bronchioles of the lung following infection, and possibly also the lacrimal gland. MAM regulation of ion channel function would be of great importance to ocular surface biology and disease, and is an area that should be a priority for investigation.

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Highlights

- *MUC1, -4, -16, -20, -21* and *-22* are expressed at the ocular surface
- Their glycoprotein products provide barrier function and act as cell surface receptors
- Biological roles are being defined in the human epithelial tissue-equivalent model
- Knockout mouse models add *in vivo* complexity
- Human genetics/genomics offers functional clues

Publication Statement

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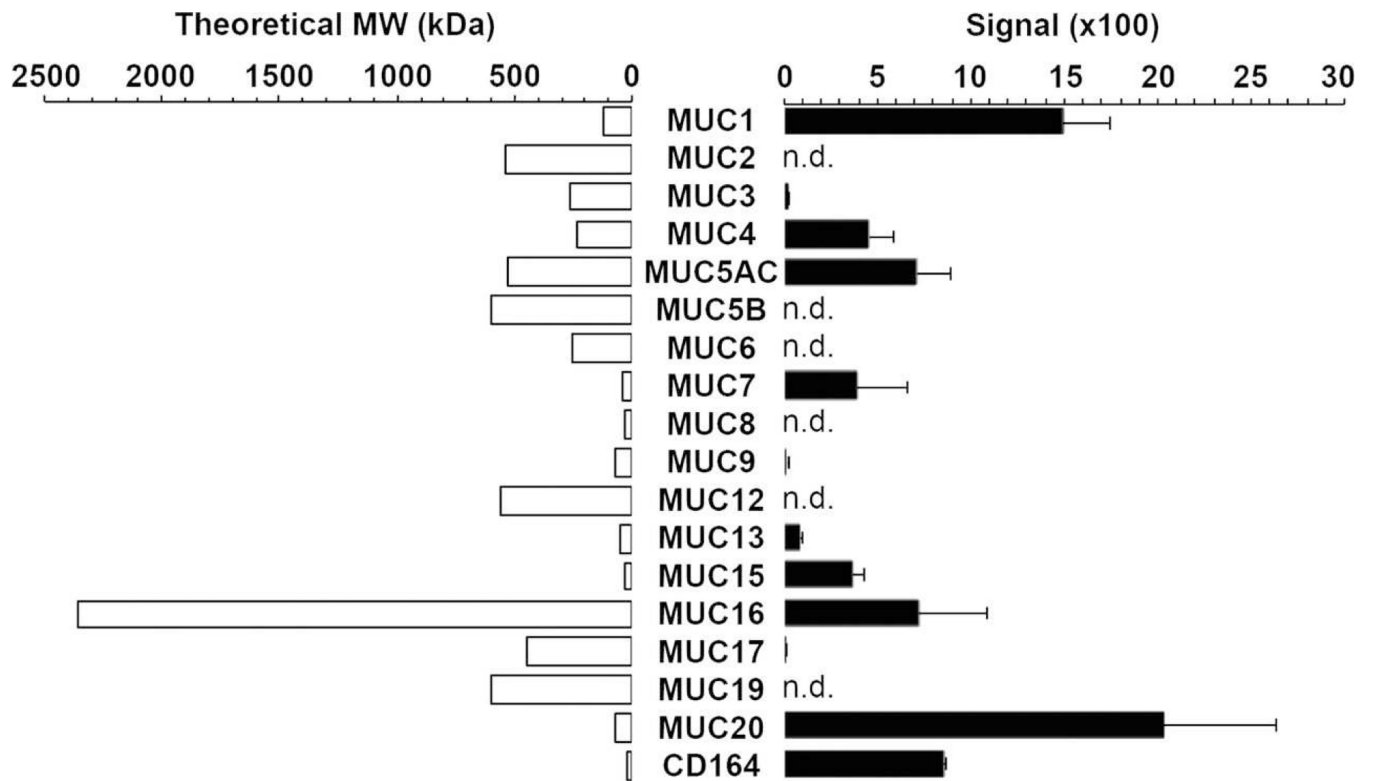


Figure 1. Mucin gene expression in human conjunctival epithelium.

Microarray analysis of impression cytology samples indicates that *MUC20* is the most highly expressed mucin gene in human conjunctiva. n.d.: not detected. *CD164* was previously designated as *MUC24*. *MUC21* and *MUC22* are not included in this analysis. From (Woodward and Argueso, 2014), with permission.

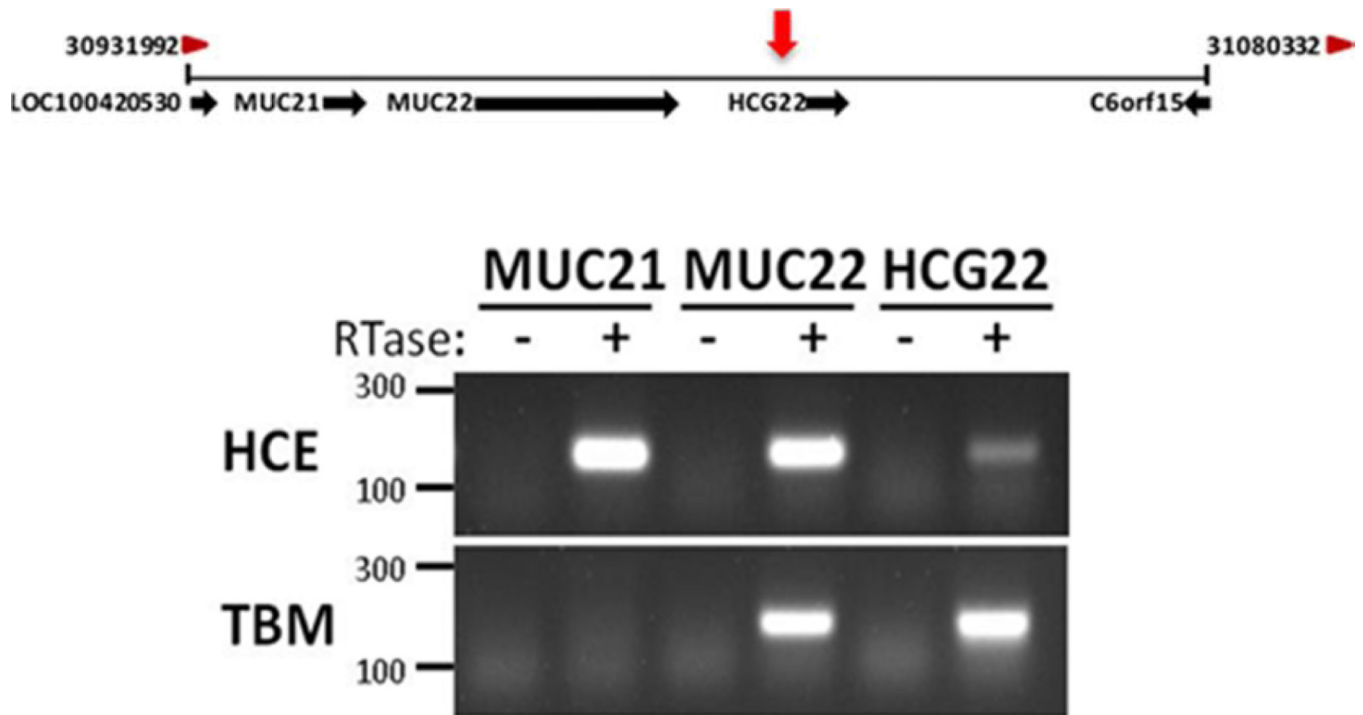


Figure 2. Location of Genes for MUC 21 and MUC22 at Chromosomal Region 6p21.32–33 and Expression in the Corneal Epithelium.

Top: Schematic of chromosomal region 6p21.32–33 from NCBI Gene depicting annotated genes surrounding an identified quantitative trait locus (QTL) for steroid-induced ocular hypertension (red arrow) in the transcriptional promotor region of HCG22.

Bottom: Total RNA was purified from cultured primary human corneal epithelial cells (HCE) and cells of the trabecular meshwork (TBM) cell line TM-1, and used for cDNA synthesis. RT-PCR using the cDNA was performed using specific primers from *MUC21*, *MUC22*, and *HCG22*; the products were resolved on a 1.5% agarose gel. Primers were designed to detect only the coding transcript. Similar results were obtained using three primary TBM cell lines (not shown).

RTase: reverse transcriptase; HCE: primary corneal epithelial cells obtained from corneal rims.

From (Jeong et al., 2015) with permission.

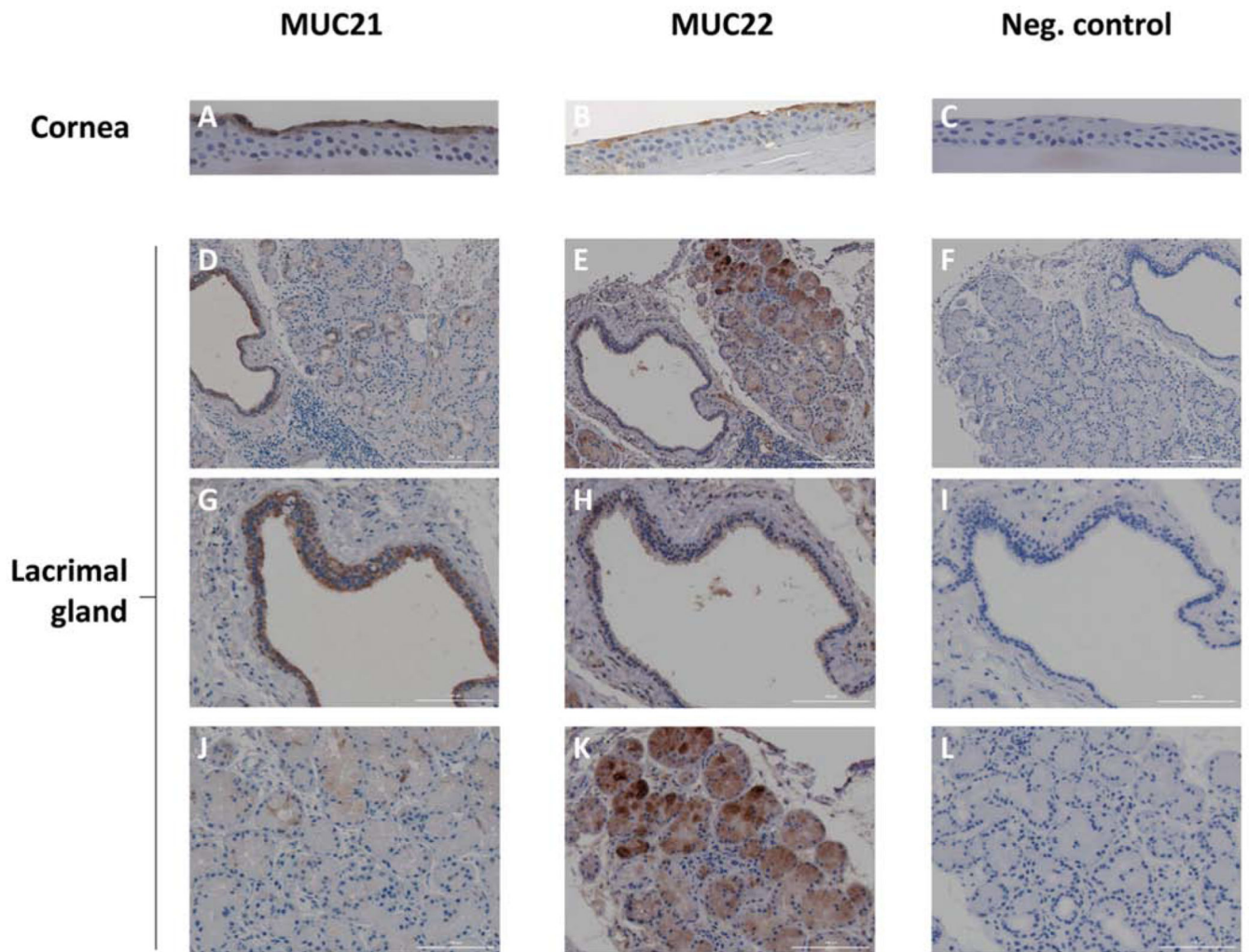


Figure 3. Immunolocalization of MUC21 and MUC22 in the Human Corneal Epithelium and the Human Lacrimal Gland.

An anterior segment isolated from a human donor eye was formalin-fixed within 24-hours post-mortem and paraffin-embedded. A formalin-fixed human lacrimal gland embedded in paraffin was obtained from the Ophthalmic Pathology Laboratory of Tufts Medical Center. Tissues cross-sections were prepared, then processed and indirectly immunostained for MUC21 or MUC22 as described (Itakura et al., 2019). The human MUC21 primary antibody was purchased from Sigma-Aldrich Corp. (St. Louis, MO). It is derived from a rabbit polyclonal antisera raised against a peptide from the human MUC21 cytoplasmic tail (561-CVRNSLSLRN TFNTAVYHPH GLNHGLGPGP GGNHGAPHRP RWSPNFWRR PVSSIAMEMS GRNS-624), then affinity-purified. The human MUC22 primary antibody was characterized in one of our labs, as described (Hijikata et al., 2011). A rabbit polyclonal antisera produced by GENENET (Fukuoka, Japan) was raised against a peptide (TPTNVIKPSGYLQP) from the human MUC22 stem region located just before the transmembrane domain, then affinity-purified. A 3,3'-diaminobenzidine (DAB) chromogen kit was used to detect secondary antibody binding (Vector Laboratories, Burlingame, CA). The negative control (Neg. control) omitted the primary antibody.

Sections were counterstained with hematoxylin. **A-C**) Cross-sections through the anterior segment focusing on immunostaining results (brown color) in the cornea epithelium. The hematoxylin counterstain is dark blue. Magnification = 40X. **D-L**) Cross-sections through the lacrimal showing immunostaining results (brown color). The hematoxylin counterstain is dark blue. **D-F**) Low magnification view (10X); **G-I**) Higher magnification (40X) focusing on a lacrimal duct; **J-L**) Higher magnification focusing on serous acini. These experimental findings have not been previously published.

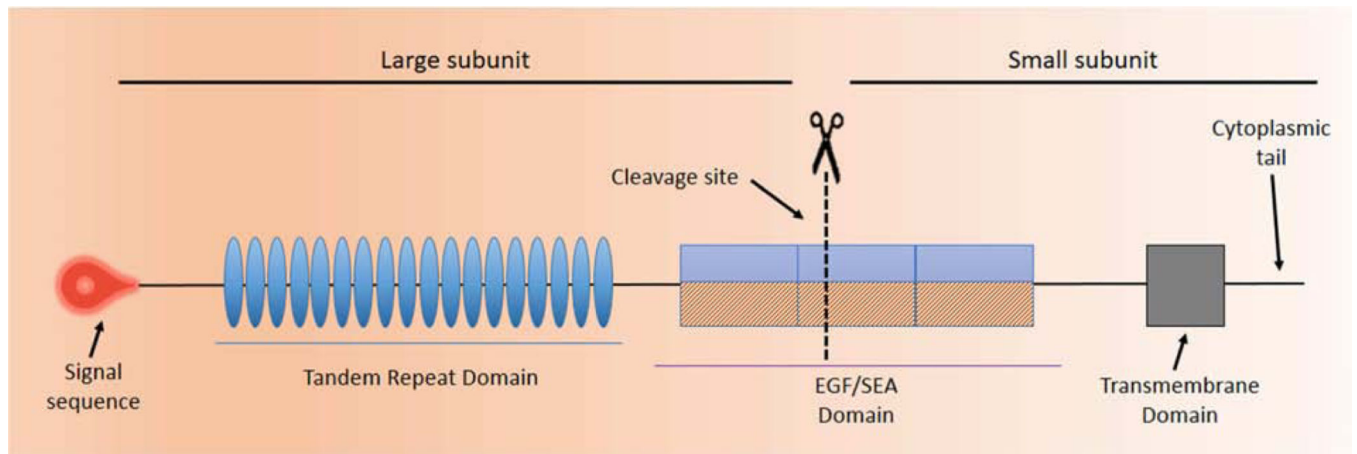


Figure 4. Prototype of a Membrane Associated Mucin (MAM).

The graphic depicts a prototypical MAM, the structure of which is similar to a classic, single-pass transmembrane immune receptor. A signal peptide motif is found at the N-terminal of the precursor polypeptide chain to enable its membrane insertion; it may be retained in the mature protein (1). The mature protein is composed of two subunits that self-associate, arising from intracellular cleavage. The large subunit is entirely extracellular and contains the VNTR. The small subunit consists of a short extracellular region, a single-pass transmembrane domain, and a cytoplasmic tail (CT). The large subunit of the MAM, together with the extracellular portion of the small subunit, comprise the extracellular domain (ED). The ED also contains conserved sequence motifs as modular elements such as the Sperm protein, Enterokinase and Agrin module (SEA) and EGF-like modules.

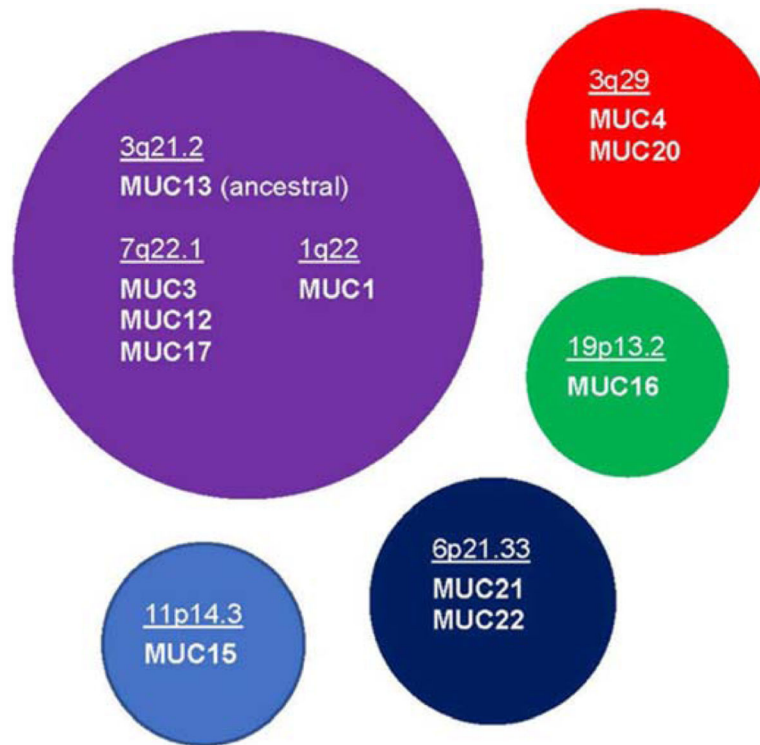


Figure 5. Proposed Evolutionary Subgroupings of Epithelial Membrane Associated Mucins (MAMs).

The best evidence is that MAMs arose largely through a process of convergent evolution, but they can be grouped into evolutionarily-related subgroups based on their genetic backgrounds, as shown in the graphic. The rationale for the groupings is discussed in the text.

Some of the information in this graphic is summarized from (Duraismy et al., 2006). The complete analysis shown here has not been previously published.

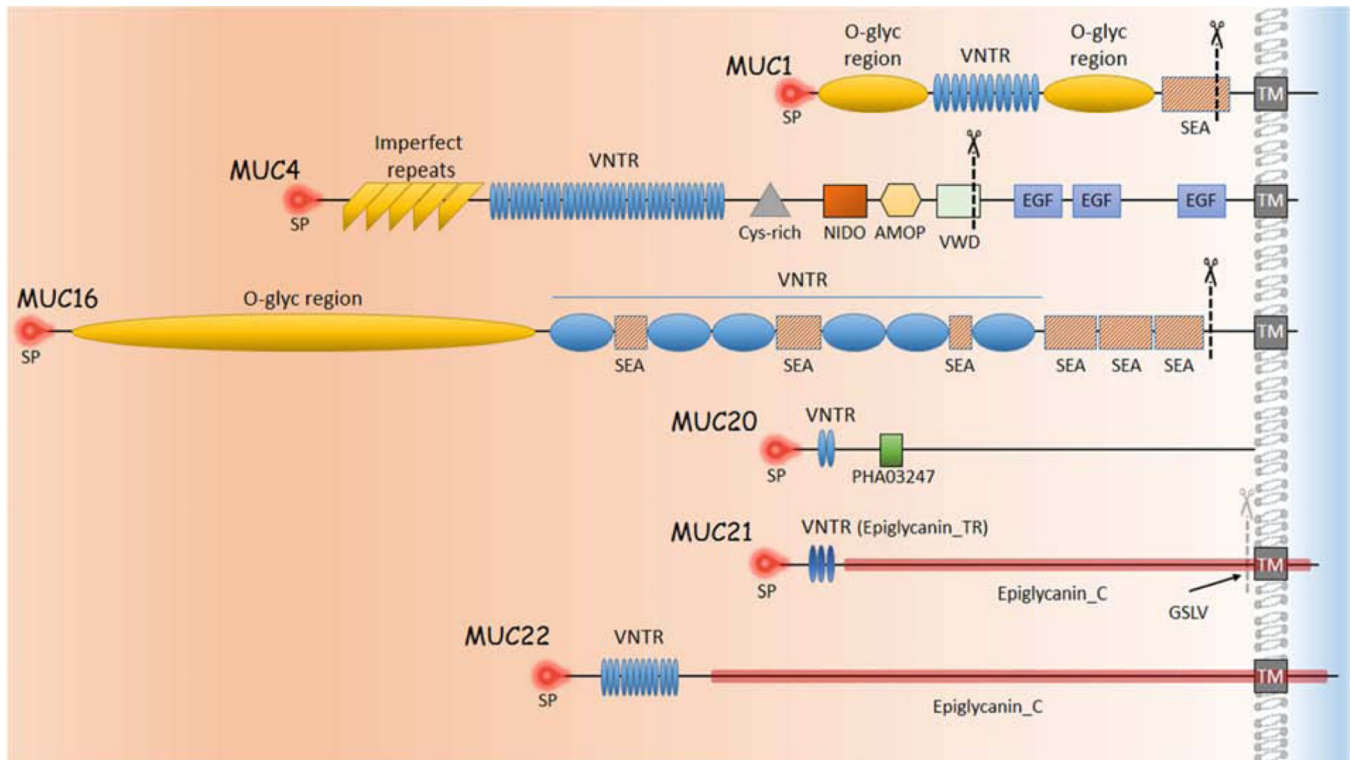


Figure 6. Modular Architecture of Ocular Surface Membrane Associated Mucins (MAMs). Shown are the extended conformations of MAM proteins prior to intracellular processing, but with the final relationship to the plasma membrane depicted. The extracellular domain of each protein is to the left of the plasma membrane. MAMs could not be drawn to scale because of extreme size differences, but an effort was made to depict relative differences in overall size, and relative location and sizes of the modular units. The signal peptides are located at the amino-terminus of each protein. The approximate intracellular cleavage sites of each mucin are indicated by scissors. MUC20 has been experimentally determined to associate with the plasma membrane, but no transmembrane domain has been identified. SP: signal peptide; TM: transmembrane domain; VNTR: Variable Number Tandem Repeats; conserved modular domains as in Table 3. GSLV: proposed cleavage site for MUC21.

nomenclature. The proposed N-terminal palmitoylation site in MUC1 and adjacent polybasic amino acid stretches in MUC1 and MUC16 are in blue text and underlined. Regions predicted to have disordered protein binding properties in human MUC21 and MUC22 are in underlined black text.

This compilation, with its new analyses, has not been previously published.

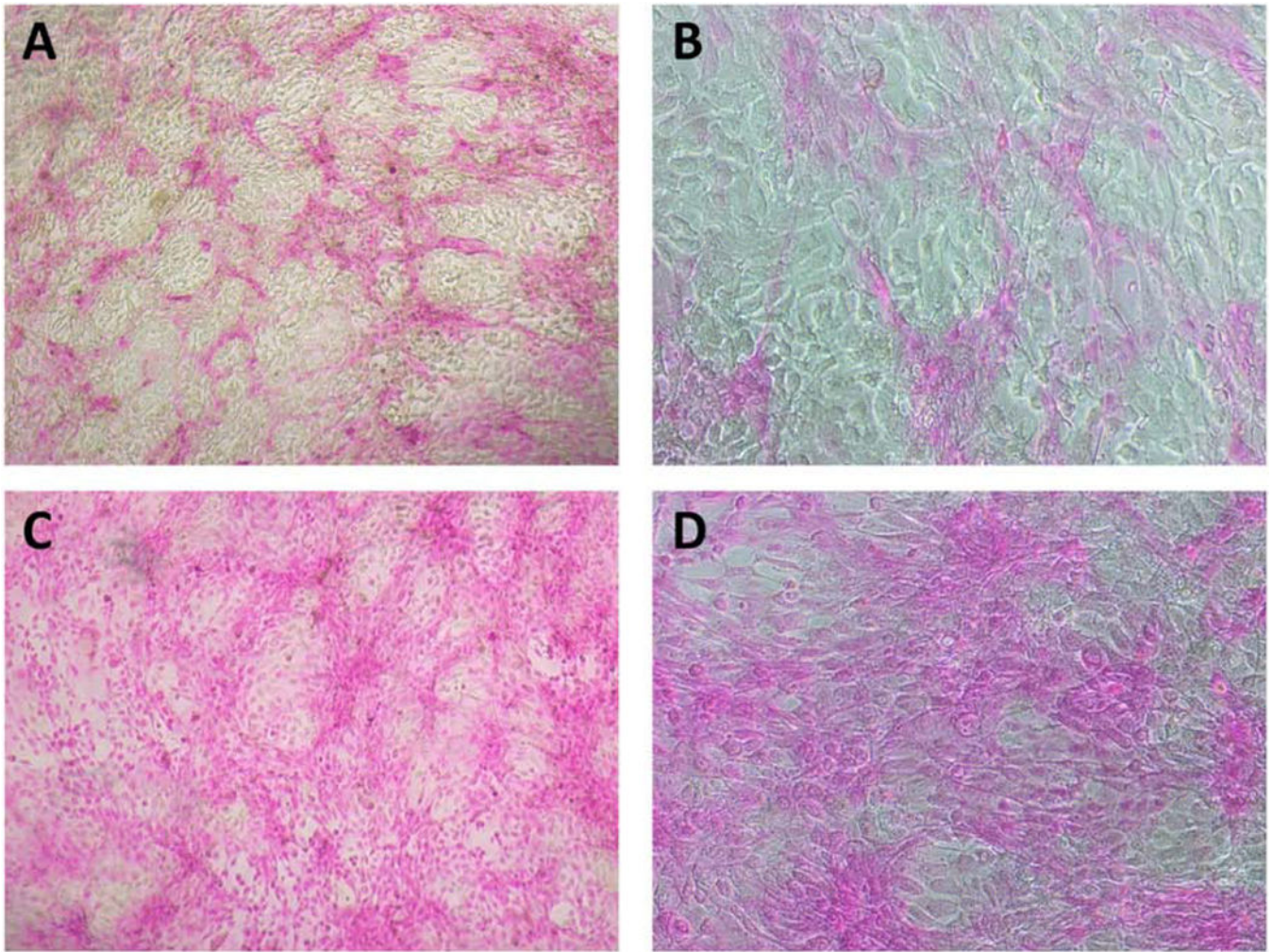


Fig 8. Application of Oxidative Stress to HCLE Epithelial-Equivalents with Mucosal Differentiation.

Islands of cells with mucosal differentiation at the surface of HCLE epithelial-equivalents exclude rose bengal (**A** and **B**). When oxidative stress is applied (10 mM tBHP in DMEM/F12 medium for 2 hours, as previously described (Webster et al., 2018)), many of these cells lose their transcellular barrier function and rose bengal penetrates (**C** and **D**). Rose Bengal staining was performed by incubating the cells 5 minutes in 0.1% Rose Bengal as previously described (Argueso et al., 2006). Magnification: **A** and **C** are 4x; **B** and **D** are 10x.

This example has not been previously published, but is similar to findings published in (Webster et al., 2018)

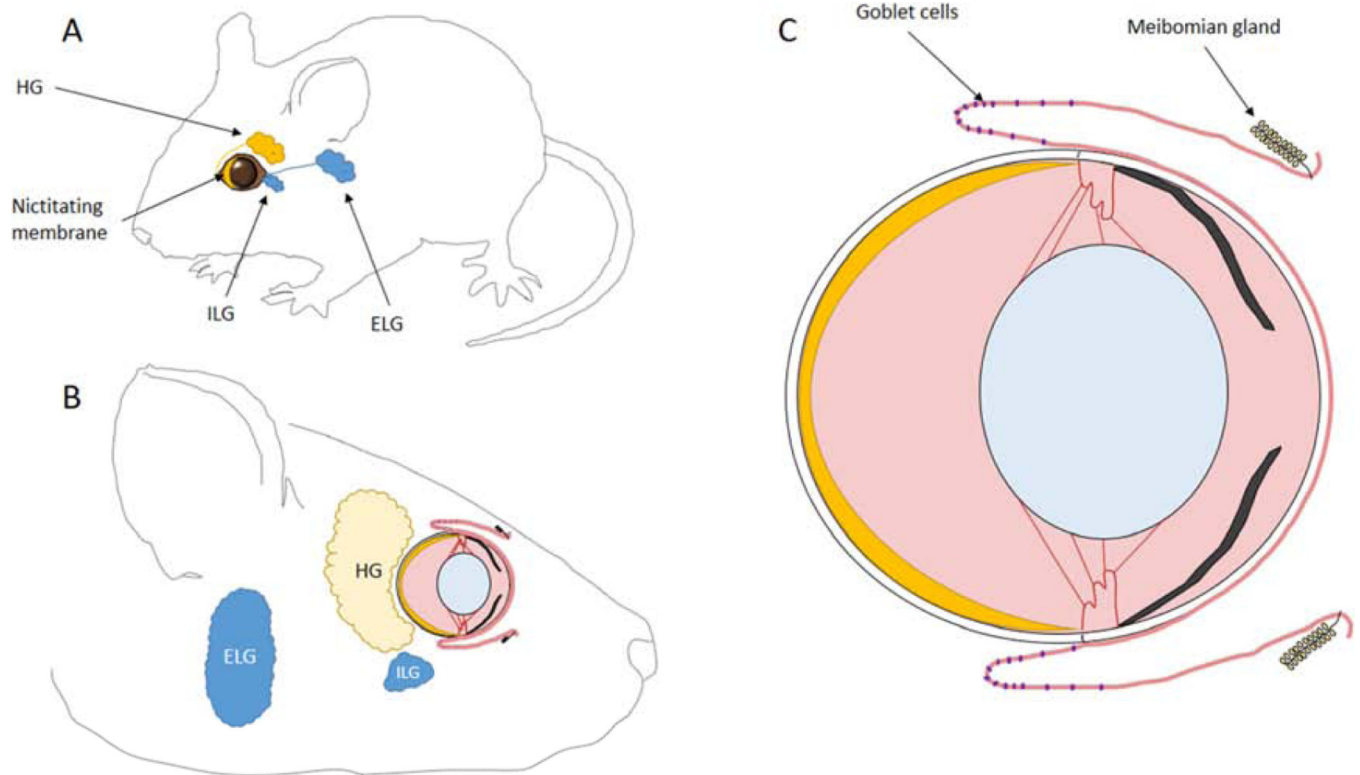


Figure 9. Schematic of the Mouse Ocular Surface System.

A) Eye in the mouse showing positioning of the glands; **B)** Larger and side view of A; **C)** Larger view of the isolated eye cross section.

ELG: Extraorbital Lacrimal Gland; ILG: Intraorbital Lacrimal Gland; HG: Harderian Gland

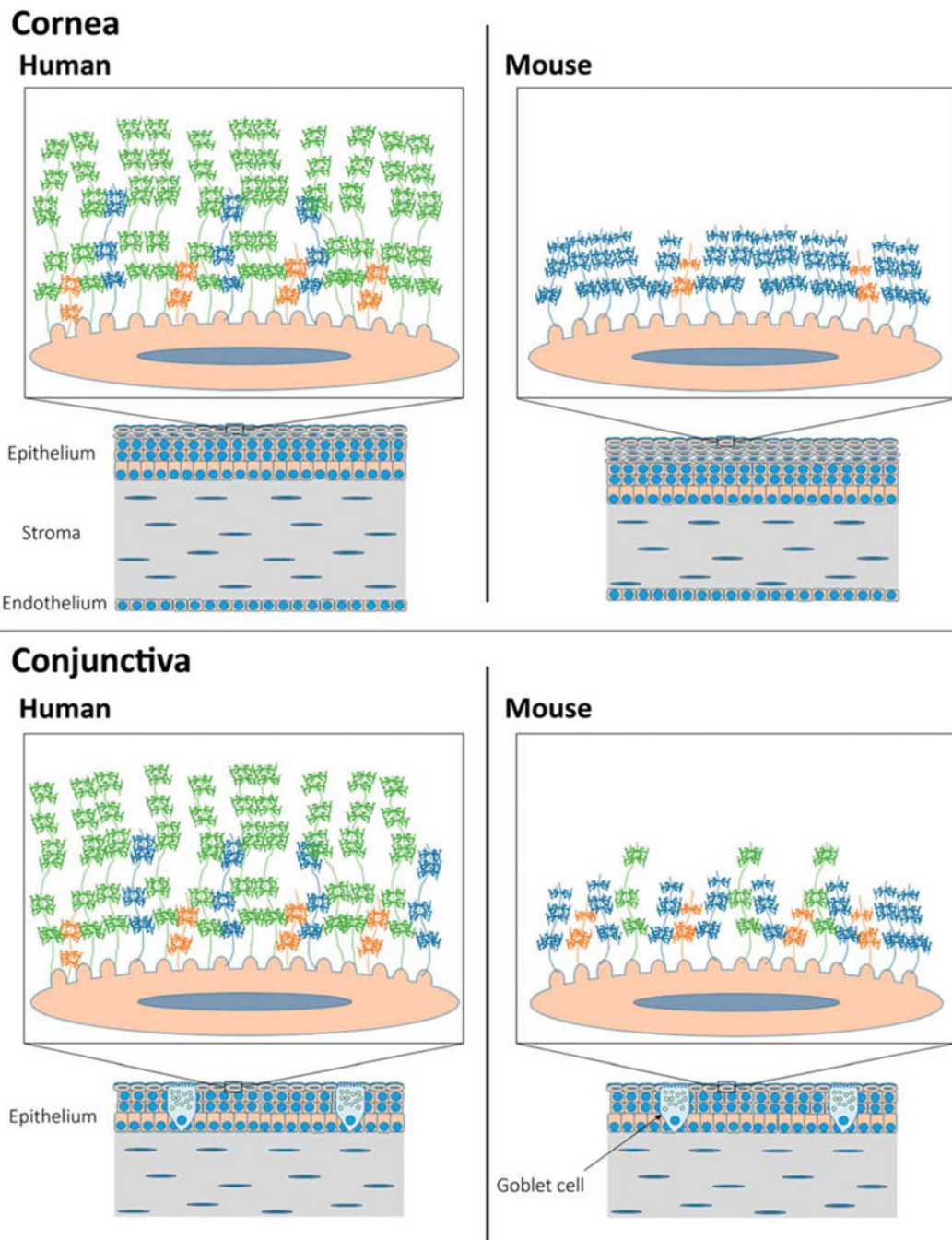


Figure 10. Comparison of MUC1, MUC4, and MUC16 Localization at the Human and Mouse Ocular Surface.

The graphics compare the corneal epithelium (top) and conjunctiva (bottom) from human (left) and mouse (right). As depicted, the corneal epithelium in mouse has more cell layers than human; the human corneal stroma is thicker than that of mouse.

The expanded insets depict a single apical epithelia cell from the corneal or conjunctival epithelium of mouse or human, showing the surface microplacae. With the EDs of MAMs MUC1, MUC4, and MUC16 projecting outward into the tear film. The EDs of the two longest MAMs, MUC16 and MUC4, are substantially shorter in mouse than human. MUC4

appears to substitute for MUC16 on the corneal epithelium of mouse, which further reduces the overall length of MAM EDs on the corneal epithelium.
MUC1: orange; MUC4: blue; MUC16: green.

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Table 1:

Human MUC Gene Family

Ordered by chromosomal location

Gene Symbol	Cytogenetic Band	Expressing Tissue(s) at the Ocular Surface; Protein Presence in Tears
<i>Secreted Mucins (gel-forming)</i>		
<i>MUC2</i>	11p15.5	conjunctiva (RT-PCR only) (^{1,2}); tears (low level) (³)
<i>MUC5AC</i>	11p15.5	Conjunctiva (^{1,2,4,5}); goblet cells (^{3,5}); lacrimal duct goblet cells (⁶); tears (³)
<i>MUC5B</i>	11p15.5	lacrimal gland (RT-PCR only) (⁷); not in tears (³)
<i>MUC6</i>	11p15.5	
<i>Secreted Mucins (soluble)</i>		
<i>OVGP1 (MUC9)</i>	1p13.2	
<i>MUC7</i>	4q13.3	conjunctival epithelia (^{2,4,7}); lacrimal gland (⁷); not in tears (³)
<i>MUC19</i>	12q12	
<i>MUC8</i>	12q24.33	
<i>Membrane-Associated Mucins</i>		
<i>MUC1</i>	1q22	corneal & conjunctival epithelia (^{2,4,8}); lacrimal gland (^{6,7}); tears (³)
<i>MUC13</i>	3q21.2	conjunctival epithelium (RT-PCR only) (^{2,4})
<i>MUC4</i>	3q29	conjunctiva, much less in corneal epithelium (^{2,4,5,9}); lacrimal gland (⁷); tears (³)
<i>MUC20</i>	3q29	corneal & conjunctival epithelia (⁴); not in tears (⁴)
<i>EMCN (MUC14)</i>	4q24	
<i>MUC21</i>	6p21.33	corneal epithelium and lacrimal gland (this paper)
<i>MUC22</i>	6p21.33	corneal epithelium and lacrimal gland (this paper)
<i>MUC3B</i>	7q22	
<i>MUC3A</i>	7q22.1	
<i>MUC12</i>	7q22.1	
<i>MUC17</i>	7q22.1	conjunctival epithelium (RT-PCR only) (²)
<i>MUC15</i>	11p14.3	conjunctival epithelium (RT-PCR only) (^{2,4})
<i>MUC16</i>	19p13.2	corneal & conjunctival epithelia (^{2,4,10}); mucin granules of conjunctival goblet cells (¹¹); lacrimal gland (¹²); tears (³)

Citations

1. (McKenzie et al., 2000)
2. (Corrales et al., 2009)
3. (Spurr-Michaud et al., 2007)
4. (Woodward and Argueso, 2014)
5. (Inatomi et al., 1996)
6. (Paulsen et al., 2004)

7. (Jumblatt et al., 2003)

8. (Inatomi et al., 1995)

9. (Pflugfelder et al., 2000)

10. (Argueso et al., 2003)

11. (Gipson et al., 2016)

12. (Jager et al., 2007)

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Table 2:

Human Epithelial MAMs Ordered by Polypeptide Length

Symbol	Amino acids	Predicted backbone mass	NCBI Protein database accession number	Isoforms
MUC16	14,507	1,519 kDa	NP_078966.2	14
MUC4	7,418	734 kDa	NP_001309397	4
MUC12	5,335	543 kDa	NP_00157934.1	1
MUC17	4,493	452 kDa	NP_001035194.1	2
MUC3A	3,323	345 kDa	NP_005951.1	6
MUC22	1,786	175 kDa	NP_001309398.1	3
MUC20	709	72 kDa	NP_001269435.1	4
MUC21	626	60 kDa	NP_001309300.2	3
MUC13	512	55 kDa	NP_149038.3	1
MUC1	484*	50 kDa	NP_001191215.1	20
MUC15	361	39 kDa	NP_001128563.1	3

Expression of MAMs in darker grey (MUC12 and MUC3A) has not been detected at the ocular surface; expression of MAMs in lighter gray (MUC17, MUC13) are documented only by RT-PCR

Protein data derived from the NCBI Protein database; listed here is the amino acid number of the longest isoform identified, with its accession number; a longer isoform of MUC1 (1255 amino acids) is listed in the UniProtKB database

Estimated molecular weight of the protein backbone mass was computed using: https://web.expasy.org/compute_pi/

* The canonical MUC1 protein listed in the UniProt database is much longer, at 1255 amino acids (discussed more in the text)

Table 3:

Conserved Motifs Found in Human MAMs Expressed at the Ocular Surface

Motif	Definition	MAMs with the Motif
SEA	Sperm protein, Enterokinase and Agrin module. Regulates or binds carbohydrate side chains	MUC1, MUC13, MUC16, MUC17
KdpC	K ⁺ -transporting ATPase, c chain module; interacts with KdpA subunit to assemble and stabilize the Kdp complex	MUC1
AMOP	Adhesion-associated domain	MUC4
NIDO	Extracellular domain of unknown function in nidogen (entactin) and hypothetical proteins	MUC4
VWD	Von Willebrand factor type D domain	MUC4
EGF-like	Cysteine-rich EGF-like modules	MUC3, MUC4, MUC12, MUC13, MUC17
PHA03247	Large tegument protein UL36, found in <i>Herpes simplex</i> virus, provisional	MUC20
Epiglycanin_TR	Tandem-repeating region of mucin, epiglycanin-like	MUC21
Epiglycanin_C	Non-tandem repeat portion of ED, including cleavage site, transmembrane domain and CT	MUC21, MUC22

All except the EGF-like motif are identified in the individual gene profiles in the NCBI Gene database

Table 4:

Human Epithelial MAMs Ordered by Length of the CT

Symbol	Amino acid number
MUC22	92
MUC17	89
MUC15	74
MUC12	74
MUC3A	73
MUC1	72
MUC13	69
MUC21	66
MUC16	31
MUC4	22
MUC20	N/A

Transmembrane domain predicted by use of the TMPred tool (Hofmann and Stoffel, 1993) on the ExPASy Bioinformatics Resource Portal (Artimo et al., 2012)

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Table 5:

Comparison of Human and Mouse Epithelial MAM Genes Ordered by Human Chromosomal Locus

Human			Mouse		
Symbol	Locus	Exons	Symbol	Locus	Exons
<i>MUC1</i>	1q22	5	<i>Muc1</i>	3 F1	7
<i>MUC13</i>	3q21.2	12	<i>Muc13</i>	16 B3	13
<i>MUC20</i>	3q22	4	<i>Muc20</i>	16 B3	5
<i>MUC4</i>	3q22	25	<i>Muc4</i>	16 B3	25
<i>MUC21</i>	6p21.33	3	<i>Muc21</i>	17 B1	3
<i>MUC22</i>	6p21.33	5	<i>Muc22</i>	N/A	N/A
<i>MUC3</i>	7q22.1	13	<i>Muc3</i>	5 G2	14
<i>MUC12</i>	7q22.1	12	<i>Muc12</i>	N/A	N/A
<i>MUC17</i>	7q22.1	13	<i>Muc17</i>	N/A	N/A
<i>MUC15</i>	11p14.2	5	<i>Muc15</i>	2 E3	4
<i>MUC16</i>	19p13.2	88	<i>Muc16</i>	9 A2	89

Table 6:

Comparison of Human and Mouse Epithelial MAMs Ordered by Human Protein Backbone Length

Human		Mouse			
Symbol	Amino Acids	Symbol	Amino Acids	Accession number	Isoforms
MUC16	14,507	Muc16	8,817	XP_911240936.1	3
MUC4	7,418	Muc4	3,470	NP_536705.3	1
MUC12	5,335	Muc12	N/A	N/A	N/A
MUC17	4,493	Muc17	N/A	N/A	N/A
MUC3	3,313	Muc3	1,802	XP_006504604.2	2
MUC22	1,786	Muc22	N/A	N/A	1
MUC20	709	Muc20	688	NP_001139346.1	2
MUC21	626	Muc21	1,606	NP_001231583.1	N/A
MUC13	512	Muc13	573	NP_034869	3
MUC1*	484	Muc1	631	NP_038633.1	1
MUC15	361	Muc15	331	NP_766567.1	2

Listed here is the amino acid count of the longest isoform identified in the NCBI Gene database for each MAM gene

*The canonical MUC1 protein listed in the UniProt database is much longer, at 1255 amino acids (discussed more in the text)

Table 7.

Comparison of MUC16 Length and Nictitans Coverage of the Ocular Surface across Primate Species Ranked by Evolutionary Distance from Human

Genus and Species	Family	Exon Count	Amino Acid Count	Isoforms	Nictitans Coverage
Human (<i>Homo sapiens</i>)	Great Apes	88	14,507	14	vestigial
Chimpanzee (<i>Pan satyrus</i>)		110	14,498	2	vestigial
Western Gorilla (<i>Gorilla gorilla</i>)		70	14,076	1	1/10
Crab-Eating Macaque (<i>Macaca fascicularis</i>)	Old World Monkey	75	14,346	1	1/8
Pig-Tailed Macaque (<i>Macaca nemestrina</i>)		90	14,035	1	1/8
Green Monkey (<i>Cercopithecus aethiops</i>)		84	14,188	1	1/5
White Tufted-Ear Marmoset (<i>Callithrix jacchus</i>)	New World Monkey	59	5,635	1	1/3.5
Bolivian Squirrel Monkey (<i>Saimiri boliviensis</i>)		100	4,002	1	
Common Squirrel Monkey (<i>Saimiri sciureus</i>)					1/3
Chinese Tree Shrew (<i>Tupaia chinensis</i>)	Treeshrew	75	11,696	1	
Tree Shrew (<i>Tupaia glis</i>)					1/2
Grey Mouse Lemur (<i>Microcebus murinus</i>)	Lemur	101	8,928	1	
Mongoose Lemur (<i>Lemur mongoz</i>)					1/2
Small-Eared Galago (<i>Otolemur garnettii</i>)	Bush baby	52	3,345	1	
Potto (<i>Perodicticus potto</i>)	Loris				1

Listed here is the amino acid count of the longest isoform identified in the NCBI Gene database.

The relative sizes of the nictitans are expressed as the ratios of total eye surface area to nictitans coverage when the membrane was extended laterally with the aid of dissecting forceps.

Table 8.

MAM Knockout Mouse Phenotypes

Gene	System	Phenotype	Citation
Muc1	Cancer	Reduced breast tumor growth, increased tumor cell resistance to chemotherapy drugs	(Spicer et al., 1995)
		Reduced pancreatic tumor progression and metastasis	(Besmer et al., 2011)
		Reduced pancreatic tumor resistance to chemotherapy	(Nath et al., 2013)
		Increased lung adenocarcinoma multiplicity; increased EREG production that activates the EGFR pathway for lung carcinogenesis	(Xu et al., 2017)
	Myeloid	Increased differentiation of bone marrow progenitors into myeloid-derived suppressor cells	(Nagaraj et al., 2009)
	Gastrointestinal	Increased intestinal susceptibility to bacterial infection due to local deficiency of Muc1	(McAuley et al., 2007)
		Increased systemic spread of orally delivered <i>Campylobacter jejuni</i>	(McAuley et al., 2007)
		Lethality of <i>H. Pylori</i> infection	(McGuckin et al., 2007) (Ng et al., 2016; Ng and Sutton, 2016)
		increased inflammatory response to pathogen exposure, NOD1 and Toll-like receptor ligands; increased chemokine secretion in response to TNFA	(Sheng et al., 2013)
	Respiratory	Dexamethasone resistance in a model for chronic obstructive pulmonary disease	(Milara et al., 2018)
		Increased morbidity and mortality due to influenza A viral infection	(McAuley et al., 2017)
		Worsening of inflammation and fibrosis in a model for interstitial lung disease	(Kato et al., 2017)
	Kidney	Worsening of kidney damage and failed recovery associated with reduced transcription factor HIF1A activation in an ischemia-reperfusion injury model; at later time points, this effect is reversed	(Pastor-Soler et al., 2015) (Gibier et al., 2017)
	Ocular Surface	Spontaneous ocular surface infection	(Kardon et al., 1999)
		No ocular surface phenotype	(Danjo et al., 2000)
Muc4	Gastrointestinal	Reduced susceptibility to colon inflammation; reduced infiltration by F4/80(+) macrophages; reduced IL1B and TNFA expression	(Das et al., 2016)
Muc13	Gastrointestinal	Mild focal neutrophilic inflammation in intestines of aged mice; increased susceptibility to inflammation in a colon inflammatory mode; increased infiltration by F4/80(+) macrophages; increased IL1B and TNFA expression	(Sheng et al., 2011)
		Decreased chemokine secretion in response to TNFA in intestine; anti-inflammatory response to pathogen exposure, NOD1 and Toll-like receptor ligands	(Sheng et al., 2013)
Muc16	Reproductive	Increased male reproductive efficiency	(Cheon et al., 2009)
	Gastrointestinal		
	Ocular Surface	Spontaneous ocular surface inflammation; altered epithelial cell dynamics; increased rate of ocular surface epithelial regeneration	(Shirai et al., 2014)

Phenotypes that are opposite of expected are in blue text