Generating an *in vitro* gut model with physiologically relevant biophysical mucus properties

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Abstract:

Introduction: Gastrointestinal (GI) in vitro models have received lasting attention as an effective tool to model drug and nutrient absorption, study GI diseases, and design new drug delivery vehicles. A complete model of the GI epithelium should at a minimum include the two key functional components of the GI tract: mucus and the underlying epithelium. Mucus plays a key role in protecting and lubricating the GI tract, poses a significant barrier to orally administered therapies and pathogens, and serves as the microbiome niche microenvironment. These functions are reliant on the biophysical material properties of the mucus produced, including viscosity and pore size.

Methods: In this study, we generated *in vitro* models containing Caco-2 enterocyte-like cells and HT29-MTX goblet-like cells and determined the effects of coculture and mucus layer on epithelial permeability and biophysical properties of mucus using multiple particle tracking (MPT).

Results: We found that mucus height increased as the amount of HT29-MTX goblet-like cells increased. Additionally, we found that increasing the amount of HT29-MTX goblet-like cells within culture corresponded to an increase in mucus pore size and mucus microviscosity, measured using MPT. When compared to *ex vivo* mucus samples from mice and pigs, we found that an 80:20 ratio of Caco-2:HT29-MTX coculture displayed similar mucus pore size, microviscosity, and elastic modulus to those of porcine ileum.

Conclusions: These coculture models could be valuable tools in simulating the mucus barrier and mucus microenvironment and can be utilized for a variety of applications including the study of GI diseases, food absorption, or therapeutic development.

Introduction

Mucus is the first line of defense to the outside world, covering our lungs, eyes, gastrointestinal tract, and urogenital tract. Mucus is a viscoelastic hydrogel-like network composed of water and mucins that effectively traps pathogens and foreign particulates, preventing them from harming or infecting the underlying epithelial cells. Mucins are high molecular weight glycoproteins, containing hydrophilic and hydrophobic domains that link and form a mesh with pores that can vary considerably between species of origin[1-4] and fall within the range of 100-500 nm[5,6]. In addition to physically trapping larger materials, the hydrophobic domains and negatively charged glycosylation on mucins entrap positively charged and hydrophobic materials[7]. In the gastrointestinal tract (GI) mucus serves as a lubricant for food, and as a barrier against pathogens, digestive enzymes and acids, digested food particles, microbial by-products, food-associated toxins. Mucus also serves as the microenvironment for the commensal GI microbiome[8,9]. Understanding GI mucus structure is particularly important, as it is not only affected by disease, but can also modulate drug absorption – a key area of study to better design orally-delivered medications. However, it is difficult to access human GI tissues and thus access to human mucus samples has been extremely limited. In the last two decades, many *in vitro* models of the GI tract have been developed to both address questions about GI physiology, and to study therapeutic absorption and toxicity to improve drug and drug delivery designs.

The most traditional GI *in vitro* models use Caco-2 cells, an immortalized human colorectal adenocarcinoma cell line. These cells grow into confluent monolayers, differentiate into polarized enterocyte-like cells, and have been used extensively to screen for the permeability and toxicity of drugs[10-12]. Caco-2 Transwell® models have been shown to recapitulate the transport of insulin, calcitonin, and exenatide that is found in *ex vivo* models[13]. However, a main criticism of the use of these Caco-2 models[13] is that, contrary to using the established 21-day culture period typically used to generate Caco-2 models, many reports cultured models for only 3-5 days prior to performing experiments, not allowing for differentiation into more representative GI epithelial cells[14]. Indeed, culturing conditions have been shown to massively affect how Caco-2 cells behave *in vitro*. Caco-2 cell models have been shown to increase in TEER and metabolic gene expression as passage number increases[15]. Also, studies on mannitol permeability across Caco-2 monolayers have demonstrated that permeability declines over culturing time, leveling off after 21 days, suggesting complete differentiation at this point[16]. Caco-2 cell layers

have also been effective in demonstrating the toxicity of both new drug formulations, and environmental hazards, such as microplastics[17]. Collectively, Caco-2 Transwell® models have proven to be a valuable tool to model the absorptive qualities of the GI tract, but they lack the mucus layer that covers the GI epithelium which can modulate transport of materials toward it, thus affecting absorption.

Addition of a mucus layer is crucial to building physiologically relevant GI *in vitro* models[1,18]. In recent years, a new cell line has emerged, HT29-MTX, that form goblet-like cells which produce mucus. Co-culturing HT29-MTX cells with Caco-2 cells generates GI epithelial models with similar gene expression profiles to GI tissue, and results in a mucus layer on top of the epithelium[19]. Studies have examined how modifying the ratio of HT29-MTX cells to Caco-2 cells can change the properties of the epithelial layer, including the thickness of the mucus produced and the resistance across the GI model. Results of these studies have been relatively variable, generally indicating that the inclusion of HT29-MTX goblet cells results in a reduction of transepithelial resistance (TEER), and an increase in mucus height, measured using microscopy[20]. Mucus layers produced by these models have been found to be 10 – 50 µm in size[21,22], and these coculture systems have largely been used to study how mucus affects drug and nutrient bioavailability[12]. Interestingly, cocultures with 10-20% HT29-MTX cells better recapitulate iron absorption trends, despite Caco-2 cells being the main contributor of iron transporters, suggesting that the inclusion of goblet-like cells modulates enterocyte physiology[23].

While new GI *in vitro* models now usually include mucus-producing cells, characterization of the mucus in these model systems has been extremely limited. Typically, analysis of mucus from *in vitro* models consists of measuring electrical resistance measurement, MUC2 (the primary mucin found in GI mucus) protein content, mucin gene expression in goblet cells, and imaging[24,25 26]. These methods are useful for confirming the presence of mucus; however, they provide relatively little information about the biophysical properties of the mucus produced by these cultures. The biophysical properties of mucus can affect its functions as a lubricant, mesh-like sieve, and homeostatic environment for the microbiome[27]. One key method to study the biophysical properties of mucus is rheology, but the limited volume of mucus produced by 1 cm^2 or smaller Transwell® models, makes use of traditional rheometers, which require >100 µL of mucus, difficult[28]. Determining microrheology through multiple particle tracking (MPT) is a useful method to gain insight into the micron-level mechanics of mucus and can be performed using the low volumes produced by *in vitro* culture models[29,30]. MPT

has already been used to determine pore sizes in vaginal, GI, and respiratory mucus[31-33]. In this study, we generated a coculture GI epithelial model using Caco-2 enterocyte-like cells and HT29-MTX goblet-like cells. We modified the ratio of the two cell types to simulate physiologically relevant cell numbers throughout the GI tract and determined its effect on the biophysical properties of the mucus within the model. We examined traditional hallmarks including epithelial morphology, TEER, and permeability, provided biophysical analysis of the mucus layer, and compared mucus properties to that of intestinal mucus from two common animal models: mice and pigs.

Methods

Cell Culture

Caco-2 and HT29-MTX cells (ATCC) were cultured in 75-cm^2 plastic flasks (Nunc EasyFlask Thermo Scientific, Nuclon Delta Surface). Culture medium consisted of Dulbecco's Modified Eagle's Medium with 10% Fetal Bovine Serum (Gibco) for both Caco-2 and HT29-MTX lines. Coculture of Caco-2 and HT29-MTX was performed at the following ratios of Caco-2:HT29-MTX: 1:0, 9:1, 4:1, and 0:1. Cells were seeded at a total density of 375,000 cells/cm^2 in 1.0 µm pore size cell culture inserts (Falcon) in a 12 Well Tissue Culture Plate (Falcon). Cocultures were maintained for 21 days to allow Caco-2 cells to differentiate and develop enterocyte-like features. All cultures were kept at 37°C in 5% CO_2, 95% air atmosphere and regularly checked every other day to change medium. Mucus was washed from cultures using phosphate buffered saline (PBS) weekly. Transepithelial electrical resistance (TEER) was measured after one to three weeks (Millipore Sigma).

Immunofluorescence Staining

Coculture Transwell© inserts were fixed with 4.0% cold Paraformaldehyde (PFA) in PBS for 15 min. Samples were then washed 3 times with PBS and kept at 4°C. Membrane inserts were cut out and placed cells-up on microscope slides. Samples were blocked with 2% FBS in PBS for 30 minutes and then washed with PBS 3 times. Primary antibody (anti-EpCAM, anti-MUC2 abcam) was diluted 1:200 in blocking solution and applied to samples for 2 h at room temperature. Primary antibody was then removed, and the sample was washed twice with PBS containing 0.1% Tween 20 and 3 times with PBS. Secondary antibody was applied for 2 h at room

temperature (Invitrogen). Cover slips were mounted on the samples with Vectashield Antifade Mounting Medium with DAPI and sealed with nail polish prior to imaging.

Coculture Permeability

Cocultures were generated on 1.0 µm transwells and cultured for three weeks as described above. 10 µg/mL fluorescent 150 kDa Dextran or albumin (Invitrogen) was added to the top well and transport to the bottom well was measured via fluorescence (using a plate reader) hourly for up to 12 h. Fluorescence intensity was quantified and tracer transport was calculated using a standard curve. Effective permeability was estimated using the equation: $\boldsymbol{P_{eff}} = \frac{\boldsymbol{C_{lower}V_{lower}}}{\boldsymbol{tSC_{initial}}}$, where C = concentration, V_{lower} = volume of the bottom compartment, S = surface area, and T = time.

Microrheology

Non-mucoadhesive nanoparticles were generated using previously published methods[33-35]. Briefly, commercially available fluorescent 100 nm, carboxyl-modified polystyrene nanoparticles (Invitrogen) were coated with 5 kDa polyethylene glycol (PEG, Creative PEGworks) using EDC/NHS chemistry. PEG coating was confirmed using dynamic light scattering (DLS, Brookhaven Scientific). Mucus was collected from the surface of the transwells and 5 µL of nanoparticles at 1E-5 w/w%, or 1 µg/mL were added. Nanoparticle movement was captured using fluorescence microscopy with an exposure time of 30 milliseconds. Centroid tracking was performed using a custom MATLAB software and mean squared displacement (MSD) was calculated[36,37]. MSD was then used along with the Stokes-Einstein equation to calculate the viscosity: $MSD = 4Dt$, where $D = k_B T/6\pi\eta r$ and D = Diffusivity Constant, k_B = Boltzmann's Constant, r = Radius of nanoparticle, T = temperature, t = time, and η = Viscosity. From these parameters, we can also estimate the pore sizes within the mucus mesh and approximate the shear and elastic moduli of the mucus[31].

For animal studies, C57Bl/6J mice were purchased and allowed to acclimate for 14 days. Porcine intestine samples were obtained from Animal Biotech Industries (ABI, Doylestown PA). Animal tissues were harvested and 5 µL of 1.0 µg/mL nanoparticles were added to the mucus covering the epithelial surface, without freezing

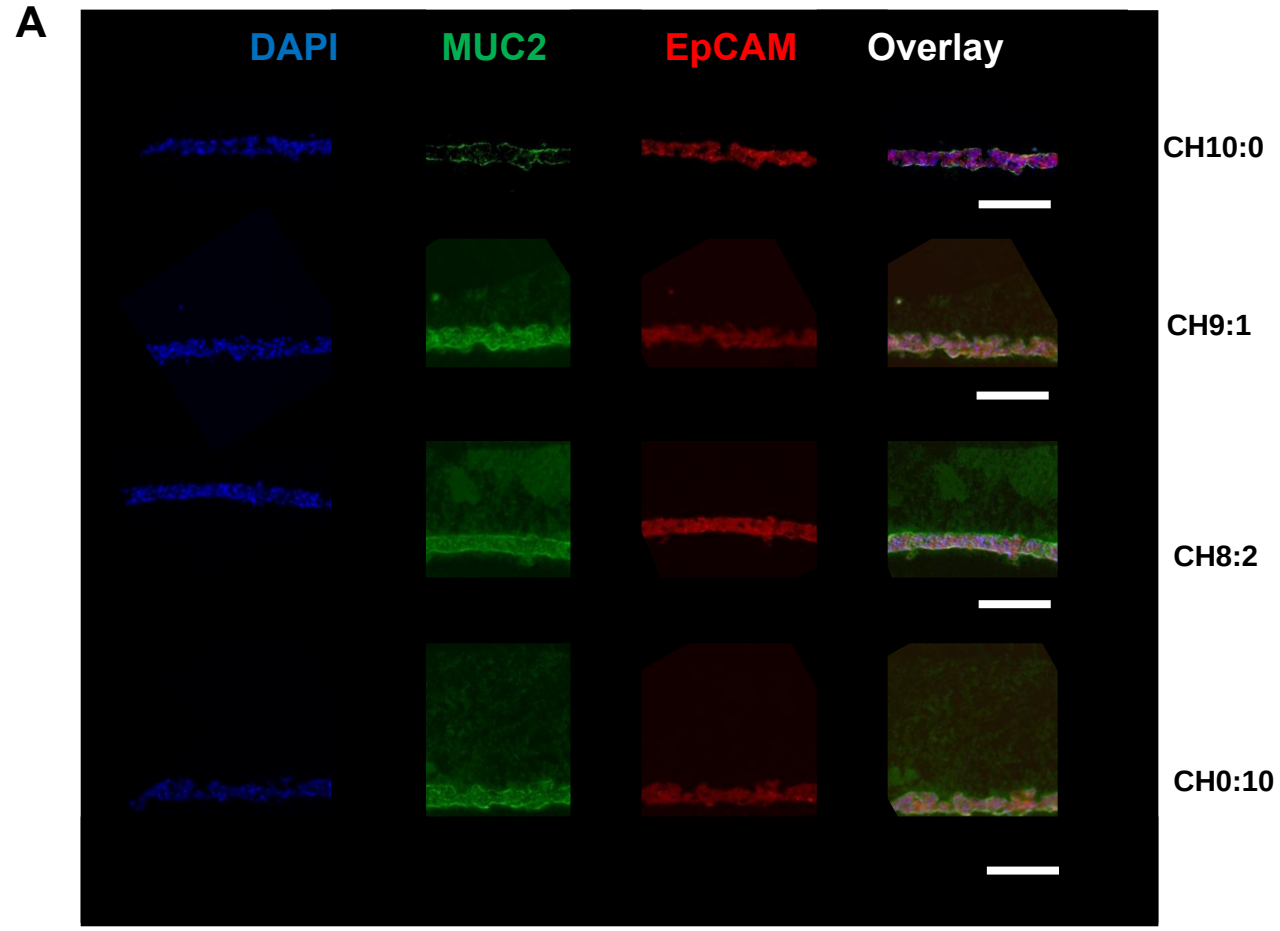
or washing tissue to preserve mucus integrity. Microrheology was performed as described above. All animal studies were approved by the UMD IACUC.

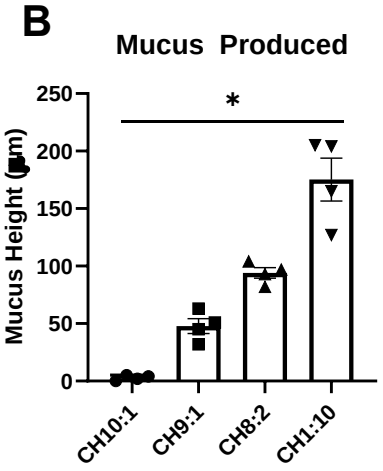
Statistics

Group analysis was performed using a 2-way ANOVA, followed by Tukey's post-test. Unpaired Student's t-test was used to examine differences between only two groups. A value of $p < 0.05$ was considered significant (GraphPad). All data is presented as mean ± standard error of the mean (SEM).

Results

We sought to generate a model GI epithelium with variable mucus by co-culturing HT29-MTX mucus producing goblet-like cells with Caco-2 enterocyte-like cells. Caco-2 cells and HT29-MTX cells were seeded at four different ratios: 1:0, 9:1, 4:1, and 0:1 Caco-2:HT29-MTX (CH10:0, CH9:1, CH8:2, CH0:10, respectively), representative of the jejunum and ileum of the small intestine [38]. Cells were cultured for 21 days on a 1.0 um Transwell© insert at confluence to allow differentiation of Caco-2 cells into enterocyte-like phenotype. We characterized junction formation of the models via EpCAM staining, as well as mucus production by staining for MUC2 (**Figure 1A**). As expected, the EpCAM signal is mostly localized to cell membranes and is most evident in monocultures of Caco-2 cells. As HT29-MTX cells are added, the signal and continuity of the EpCAM signal is reduced, but monolayer integrity is maintained, as no obvious gaps between cells can be observed. We confirmed formation of a mucus layer via MUC2 staining, which indicates that the mucus layer grows as the ratio of HT29-MTX cells in the culture is increased (**Figure 1A**). As expected, no mucus layer is apparent in the cultures containing only enterocyte-like Caco-2 cells, as these cells do not secrete mucus even though they can express MUC2 [39]. The mucus layer height is tallest on the cultures containing only HT29-MTX cells: 175 ± 37 µm, and smaller in the cocultures: 48 ± 13 µm for CH9:1, and 94 ± 10 µm for CH8:2 (**Figure 1B**). Coculture viability was close to 100% for all conditions (**Figure 1C**).





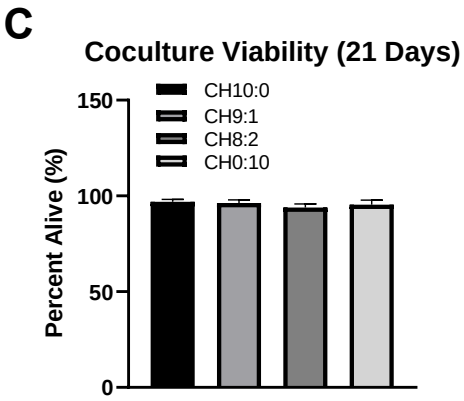


Figure 1 ***Morphology and Physiology of GI Co-Culture Models***
A) Immunofluorescence images of different co-culture conditions visualizing tight junctions (EpCAM, red), mucin (MUC2, green) and nuclei (DAPI, blue) Scale bar 80 µm. B) Quantification of mucus height C) Culture viability at three weeks. (n = 4) *p<0.05 Data ± SEM

After characterizing the morphology of the different GI cocultures, we sought to assess the effect of a mucus layer on model GI epithelial permeability. To confirm robust junction formation between epithelial and goblet cells, we monitored TEER values over three weeks, and, as expected, found that TEER increased from 330 ± 30 to 435 ± 110 mV to 508 ± 60 mV for CH10:0 and from 180 ± 20 to 258 ± 30 mV to 230 ± 40 mV for CH0:10. Both mixed cultures also increased over time, with the CH9:1 culture increasing from 370±10 to 527±30 mV to 570 ± 50 mV and the CH8:2 culture increasing from 424 ± 20 to 548 ± 20 mV to 610 ± 40 mV (**Figure 2A**). We

observed an increase in TEER with the addition of HT29-MTX cells to the culture across all time points. We also assessed solute permeability of the coculture model using fluorescent bio-inert 150 kDa dextran and albumin, a model protein. Measuring the trans-epithelial transport of the 150 kDa dextran, we found that the uniform HT29-MTX culture was the most permeable, with 12.1 ± 2.2% transport efficiency (**Figure 2A**). The other coculture conditions, CH10:0, CH9:1, and CH8:2, displayed similar transport efficiency, ranging from 8.9±0.4-9.8±0.7% after 6 hours. Albumin transport displayed similar trends as dextran transport with the HT29-MTX culture being most permeable where 5.7±3.7% of the albumin transported (**Figure 2B**). As with the dextran transport, the other coculture conditions, CH10:0, CH9:1, and CH8:2 displayed similar albumin transport efficiency, ranging from 1.7-2.7±0.8%. Using this transport data, the effective permeability of the different cocultures was calculated. The HT29-MTX culture was the most permeable to both albumin and dextran, and the other cell compositions displayed similar effective permeabilities for their respective tracer (**Figure 2D-E**).

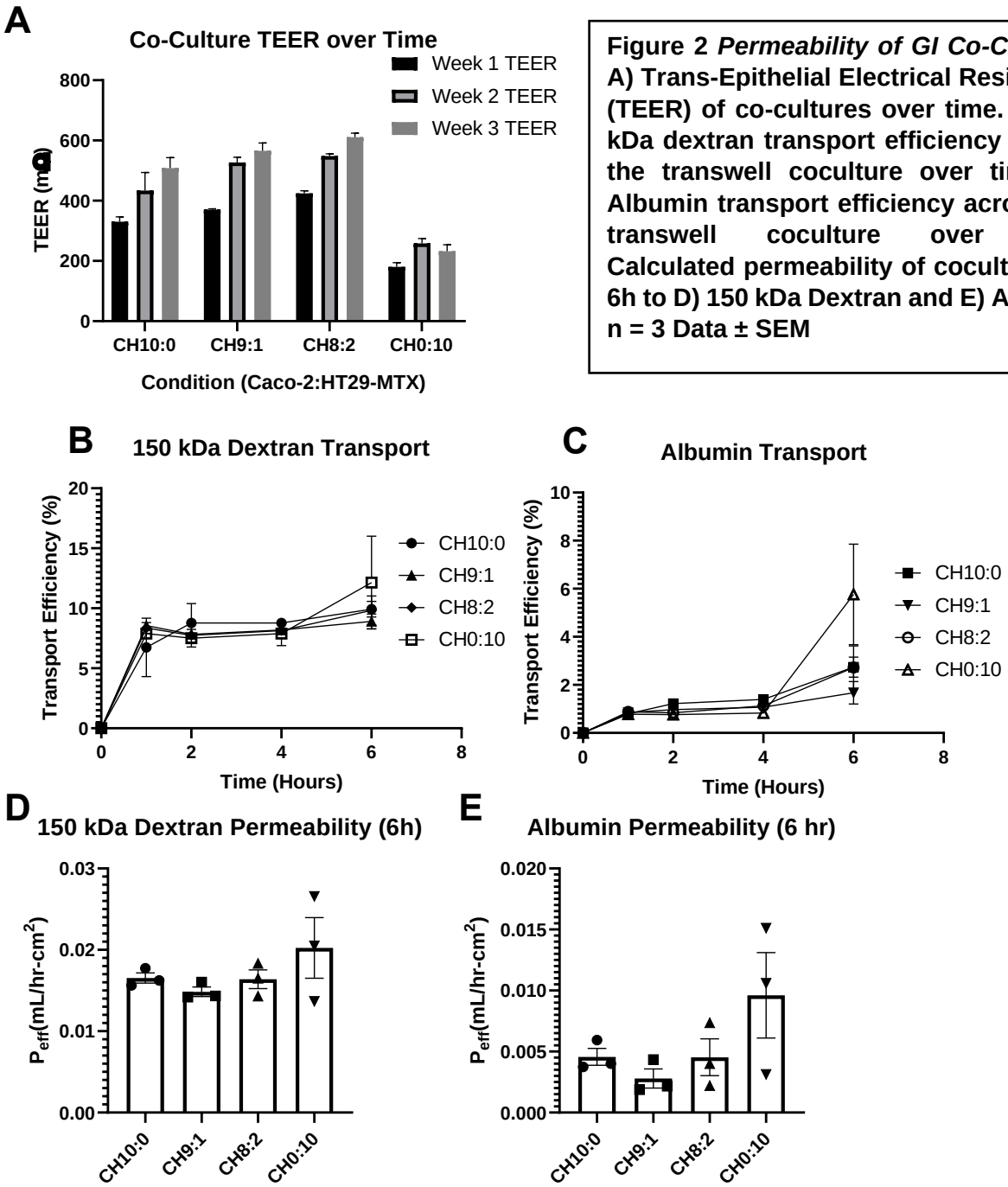


Figure 2 ***Permeability of GI Co-Cultures*** **A) Trans-Epithelial Electrical Resistance (TEER) of co-cultures over time. B) 150 kDa dextran transport efficiency across the transwell coculture over time. C) Albumin transport efficiency across the transwell coculture over time. Calculated permeability of cocultures at 6h to D) 150 kDa Dextran and E) Albumin n = 3 Data ± SEM**

Next, we characterized the rheological properties of the mucus layer in our coculture model systems using MPT. We found that MSD and diffusivity increased as the ratio of goblet cells within the culture increased. PEGylated non-mucoadhesive nanoparticles (MPP) displayed a diffusivity of 0.11 ± 0.2 μm/s^2 in CH9:1 mucus, and 0.12 ± 0.01 μm/s^2 in CH8:2 mucus. MPPs within mucus samples from goblet-cell monocultures (CH0:10) had a diffusivity of 0.13 ± 0.01 μm/s^2 (**Figure 3A**), indicating that all cultures had similar diffusivities. We found that the viscosity of the CH9:1 mucus was 0.03±0.01 Pa-s (**Figure 3B**). The mucus of the CH8:2 mucus was ~25% more

viscous than CH9:1 mucus, at 0.04±0.01 Pa-s (**Figure 3B**), while CH0:10 mucus was the most viscous at 0.045 ± 0.01 Pa-s (**Figure 3B**). We then extrapolated pore size of the mucus layers and found that there was a relatively large variation. We found that the average pore size was 440 ± 15 nm^2 in the CH9:1 mucus and 610±30 nm^2 in the CH8:2 mucus. The CH0:10 mucus had an average pore size of 620 ± 70 nm^2 (**Figure 3C**). However, pore sizes ranged from 10 to 1200 nm^2 for CH9:1 and 10 to 2800 nm^2 for CH8:2, highlighting the heterogeneity of the produced mucus layer (**Figure 3C**). Finally, we determined the elastic (storage) modulus (G') and plastic (loss) modulus (G''). The elastic modulus increased as more mucus-producing HT29-MTX cells were introduced. G' at τ=0.5s increased from 0.15 ± 0.06 Pa to 0.20 ± 0.12 Pa when the number of HT29-MTX cells was increased from 10% to 20% (CH9:1 vs. CH8:2, respectively). The monoculture of HT29-MTX goblet-like cells had the highest elastic modulus of 0.48±0.19 Pa (**Figure 3D**). This trend was mirrored when measuring G'': the plastic modulus increased as the number of HT29-MTX goblet-like cells was increased (**Figure 3E**). Interestingly, when measuring G''/G' we saw that the mucus produced by the cocultures behaved elastically (G''/G' < 1), whereas the monoculture of HT29-MTX cells was slightly plastic (G''/G' $\geq$ 1) (**Figure 3F**).

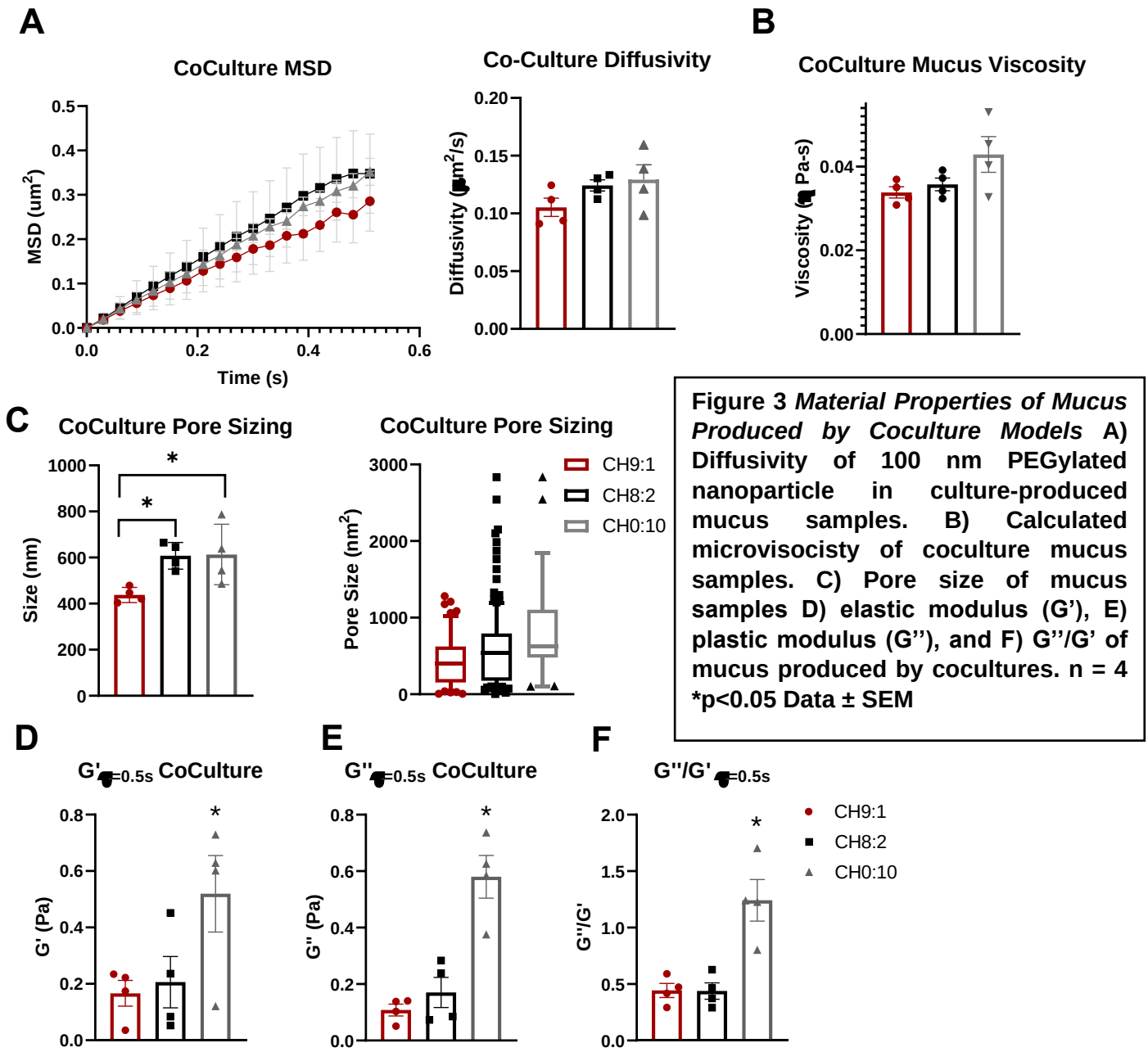


Figure 3 ***Material Properties of Mucus Produced by Coculture Models*** **A) Diffusivity of 100 nm PEGylated nanoparticle in culture-produced mucus samples. B) Calculated microvisocisty of coculture mucus samples. C) Pore size of mucus samples D) elastic modulus (G'), E) plastic modulus (G''), and F) G''/G' of mucus produced by cocultures. n = 4 *p<0.05 Data ± SEM**

To determine if the properties of the mucus produced in our *in vitro* model was representative of *in vivo* mucus, we collected GI mucus from mice and pigs, two animal models commonly used for studying the GI tract. We compared the mucus properties in both the jejunum and ileum of the small intestines. We found that MPP diffusivity was higher within the jejunum when compared to the ileum for both porcine and murine mucus samples (**Figure 4A**). MPP diffusivity dropped from 0.40 ± 0.1 to 0.20 ± 0.1 µm/s^2 for the porcine samples and 0.30 ± 0.1 to 0.19 ± 0.1 µm/s^2 for the murine mucus samples. The differences in diffusivity were mirrored with microviscosity

measurements. Porcine jejunal mucus, 0.034 ± 0.01 Pa-s, was less viscous than the ileal mucus, 0.087 ± 0.01 Pa-s. The jejunal mucus displayed similar microviscosity measurements to the CH8:2 coculture model (0.04±0.01 Pa-s). The mouse mucus viscosity was slightly higher, measuring 0.07±0.02 Pa-s in the jejunum and 0.10 ± 0.03 Pa-s in the ileum (**Figure 4B**). Using the same MPT method to measure the mucus pore sizes, we found that the mucus pores within all tissue samples and sources were smaller than those produced by our coculture models. The exception being that porcine jejunal mucus had a mucus pore measuring 390 ± 50 nm^2, similar to the mucus produced by the CH9:1 coculture model (440 ± 15 nm^2) (**Figure 4C**). Calculating the material properties of the animal mucus, we found that both were elastic in nature, evidenced by the ratio of G''/G' < 1 (**Figure 4D-F**), suggesting that the coculture models appropriately reflected *in vivo* properties. Collectively, these results indicate that there are similarities in material properties between the mucus produced by the *in vitro* models and mucus from *in vivo* animals and that *in vitro* mucus properties can be tuned to simulate *in vivo* properties by modifying culture conditions.

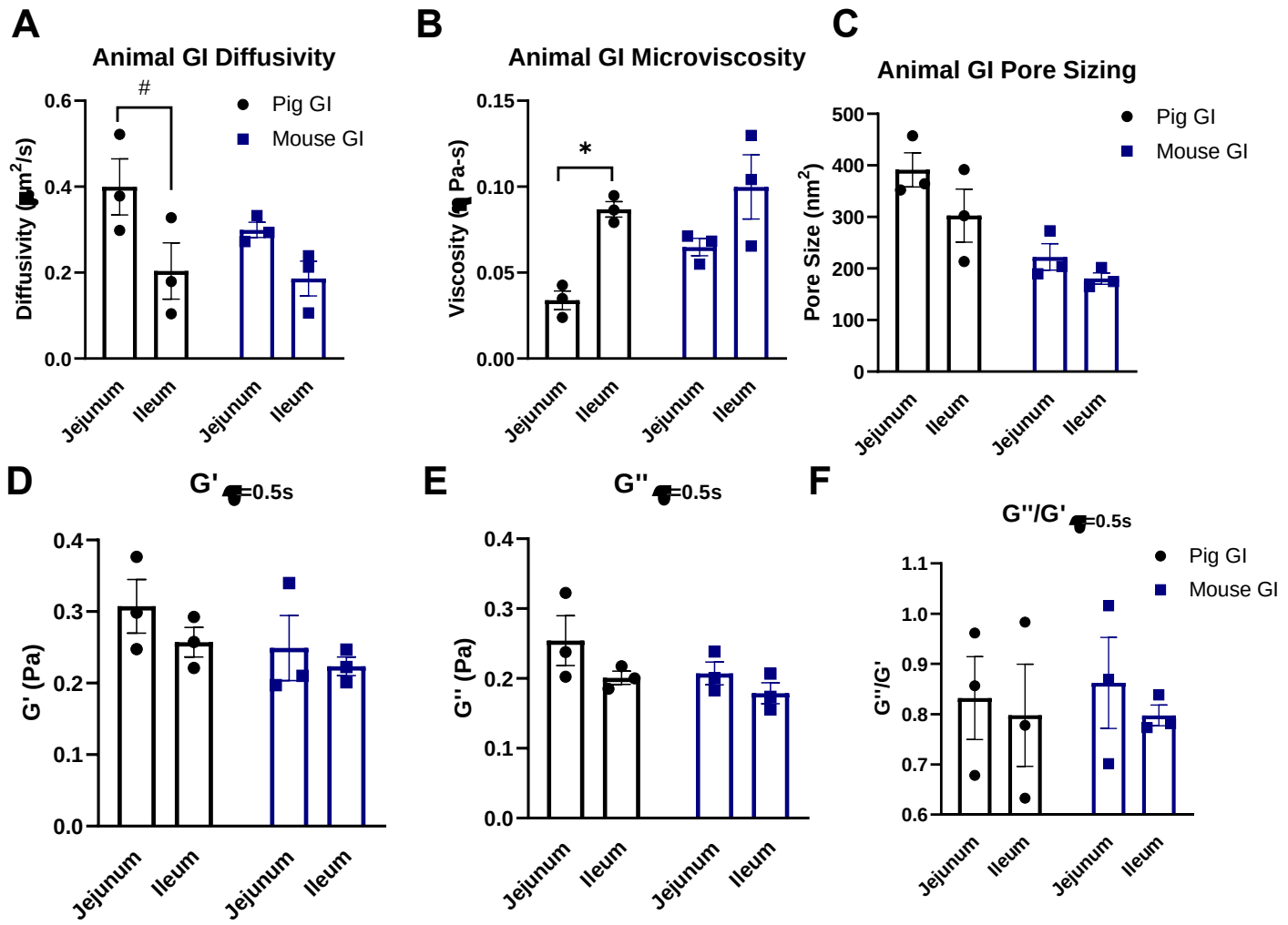


Figure 4 ***Material Properties of Mucus from In-Vivo Animal Models*** **A) Diffusivity of 100 nm PEGylated nanoparticle in collected mucus samples. B) Calculated microvisocisty of mucus samples. C) Pore size of mucus samples D) elastic modulus (G'), E) plastic modulus (G''), and F) G''/G' of mucus. n = 3 (#: p = 0.054, *p<0.05) Data ± SEM**

Discussion

In this work we established an *in vitro* model of the mucus-producing gastrointestinal (GI) epithelium and characterized the permeability of the model as well as the material properties of the mucus produced by the model. We found that increasing the number of HT29-MTX cells within our culture model resulted in more mucus production, as indicated by an increase in mucus height. The addition of HT29-MTX cells, and thus a mucus layer, resulted in an increase in TEER, but did not significantly affect the model's permeability of the albumin and 150 kDa dextran. Our microrheological analysis revealed that the mucus became more viscous and contained larger pores as the ratio of HT29-MTX cells was increased. Interestingly, we show that the mucus produced by

the coculture was distinct from the mucus produced by the HT29-MTX cells alone. The mucus produced by the cocultures was more viscoelastic than that produced by the monoculture of HT29-MTX cells. The mucus produced by the coculture models was also less viscous than that found in both the porcine and murine GI. In summary, we generated and characterized an *in vitro* model for the GI epithelium that can inform oral drug delivery strategies while providing some of the first analyses of the mucus layer produced by an *in vitro* GI model.

Studies have found that the addition of a mucus layer via coculture of HT29-MTX and Caco-2 cells does not modulate permeability of the *in vitro* model system compared to Caco-2 cells alone. Our study confirms these findings: We found that changing the ratio of cells within our culture did not change the permeability of 150 kDa dextran and albumin. Others have also reported that the addition of HT29-MTX cells does not change the permeability of chemical, macromolecule, or protein tracers in a significant way[40,41]. Interestingly, we have found that addition of HT29-MTX cells modifies formation of tight junctions between the enterocyte-like cells, which has been observed in numerous other studies and occurs because the goblet-like cells do not adequately form tight junctions themselves. However, other groups found that changing the ratios of the cell types did not alter the TEER values measured[42,43]. This is contrary to what we have observed, where the introduction of HT29-MTX cells seems to have increased the TEER of our models. This inconsistency may be caused by the thicker mucus layer in our system, which may alter the TEER readings. Overall, our system recapitulates the permeability and mucus production of previously developed *in vitro* model systems.

Mucus height changes throughout the different parts of the GI tract, which is unsurprising since the jejunum, ileum, and colon have distinct functions – food digestion/absorption and water absorption. The duodenum has the thickest mucus layer, measuring up to 500 microns, likely to prevent the incoming gastric juices and digestive enzymes from reaching the epithelial surface too quickly. In the jejunum, mucus thickness is only 150-300 microns, likely to aid in food absorption, while the mucus layer in the ileum can measure up to 500 microns in thickness [21]. These values are much greater than those recreated in classic *in vitro* models of the GI tract, where mucus thickness mostly resembles that of the firmly adherent mucus layer, ranging from 15 – 30 microns in the small intestine[3,22,24]. Our model system had mucus of 40 – 80 microns, suggesting that our system includes a thicker mucus layer than previously reported systems, which may make it more physiologically representative.

Further optimization to enhance the height of the mucus layer *in vitro* may be needed to build truly representative *in vitro* model systems.

In addition to mucus height, the material properties of the mucus change along the different parts of the intestine. Studies in mice have suggested that mouse colonic mucus has an average pore size close to 200 nm, as 200 nm, non-mucoadhesive nanoparticles had reduced diffusivity in the mucus layer[33]. Mouse small intestinal mucus, on the other hand, had a larger pore size ranging between 200-500 nm, as 200 nm nanoparticles freely diffused with the mucus gel, while 500 nm nanoparticles did not[32]. Studies using reconstituted porcine-derived mucin have estimated that the pore size of small intestinal mucus lies between 100 – 200 nm in size[44]. This has been corroborated by Yildiz et al. who observed a 20-fold decrease in particle diffusivity when nanoparticle diameter was increased from 100 nm to 500 nm, suggesting that the pore sizes within porcine mucus lie between 100 – 500 nm[45]. A confounding factor in many of these studies is that the reliance on mucus hydrogels generated from purified and heavily processed gastric mucins, instead of small and large intestinal mucins, which can lead to gels that do not necessarily recapitulate the mucin seen *in vivo*[46]. Indeed, electron micrographs of *ex vivo* mucus highlight the heterogeneity of mucus pore sizes *in vivo* and provide a visual estimation of pore size closer to 500 nm[47]. One group demonstrated that microparticles of 0.5 – 2 μm^3 were able to diffuse through murine ileal mucus samples, suggesting that mucus pore sizes could be as large as 2 μm^2 [48]. Our data shows that coculture in vitro models produce mucus with pore sizes more in line with that of estimated electron microscopy images of human mucus, a range of 50 - 250 nm[49]. Mucus pore size measurements have continued to result in seemingly conflicting data, which can largely be contributed to the myriad factors that can influence mucus permeability, including local concentrations of mucins and other macromolecules, fluid flow, presence of food materials, and diet. Collectively, these studies have suggested that pore sizes in mucus are extremely heterogeneous, which is recapitulated in our coculture models.

Mucus viscosity is key in maintaining barrier properties and is typically a function of pore size, with a tighter mesh network resulting in more viscous mucus. We found that the mucus from our cocultures had a viscosity of 0.03 - 0.04 Pa-s. This viscosity was below the reported ranges of 0.06 - 0.10 Pa-s in pig mucus, as measured via macrorheology[50]. However, differences in macro- and microrheology are common in other tissues as well. A similar study examining mucus barrier properties within different regions of the mucus layer corroborated our

measurements of mucus microviscosity. Using MPT, they found that *ex vivo*, unmodified, jejunal porcine mucus had a microviscosity in the range of 0.01-0.05 Pa-s, and remained relatively similar at different depths of the mucus layer[28,32,51]. Despite these studies, how viscosity and pore sizes vary along the small intestinal tract is still largely understudied. Comparisons between small intestine mucus and colonic mucus suggest that viscosity of mucus increases as the GI tract progresses. Indeed, a study by Swidsinski et al. indirectly measured this gradient in mucus through analysis of the microbiome components within different regions of the GI tract[52]. Using agarose gel of different viscosities, they demonstrated that bacterial shape correlated with an environmental viscosity best suited for survival and mobility. Through this, they hypothesized that the shape of bacteria in specific regions of the GI tract can predict the material properties of that region. Using this strategy, they further hypothesized that mucus viscosity increases from proximal to distal regions of the intestines. This trend is corroborated by our findings where our *ex vivo* microrheological measurements of porcine and mouse mucus indicate that viscosity increases from the jejunum to the ileum.

With inflammatory diseases at the forefront of *in vitro* modeling using Caco-2 and HT29-MTX cultures, many groups are also experimenting with adding immune cells into GI models. This is typically achieved through the addition of Raji B cells, microfold (M) cells, as well as THP-1-derived macrophages[53,54]. Ude et al. incorporated Raji B cells within a Caco-2-based model of the GI tract to generate models sensitive to copper oxide pollutants[55]. Kerneis et al. added lymphocytes from Peyer's patches to a Caco-2 GI model. Within the model, these lymphocytes behaved like M cells, transporting inert nanoparticles and bacteria[56]. Beterams et al. recently developed a triple coculture model consisting of THP-1-derived macrophages, T84 epithelial cells, and LS-174T goblet cells[57]. They found that the inclusion of immune cells resulted in an increase in mucus height from 60 to 80 µm, compared to a coculture with no macrophages, suggesting that inclusion of immune cells is crucial to create a more physiologically representative mucus height. The group further introduced model microbiota which induced pro-inflammatory cytokine production. While these studies highlight the promise of modeling both microbiota and disease interactions *in vitro*, a key next step for these models will be to include the microbiota within a mucus gel in these models. This is incredibly challenging as most microbiota require anaerobic conditions, while epithelial cultures require oxygen. However, generating mucus models with physiologically

relevant material properties could facilitate the incorporation of microbiota and allow them to behave as they would *in vivo*.

In this study we demonstrated how tuning the ratio of enterocyte and goblet cells within a GI model resulted in tissues with tunable features. We found that increasing the number of goblet cells resulted in a more resistant monolayer, while also producing mucus that was more viscous with larger pores. Being able to tune these features of the mucus microenvironment is key for engineering oral therapeutics which must cross this barrier, as well as studying microbiome interactions with its physiologically relevant microenvironment. Future GI *in vitro* models should consider the material properties of the mucus produced, as features like pore size dictate microbiome health as well as drug delivery. Models can include additions such as immune cells and microbes to provide more physiologically relevant model systems.

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