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Genetically Encoded Toolbox for Glycocalyx Engineering: Tunable Control of Cell Adhesion, Survival, and Cancer Cell Behaviors

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Abstract

The glycocalyx is a coating of protein and sugar on the surface of all living cells. Dramatic perturbations to the composition and structure of the glycocalyx are frequently observed in aggressive cancers. However, tools to experimentally mimic and model the cancer-specific glycocalyx remain limited. Here, we develop a genetically encoded toolkit to engineer the chemical and physical structure of the cellular glycocalyx. By manipulating the glycocalyx structure, we are able to switch the adhesive state of cells from strongly adherent to fully detached. Surprisingly, we find that a thick and dense glycocalyx with high *O*-glycan content promotes cell survival even in a suspended state, characteristic of circulating tumor cells during metastatic

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ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsbiomaterials.7b00037.

Full details on cloning and supporting figures (PDF)

Time-course of Muc1 CT expression (AVI)

Attachment followed by detachment (AVI)

Division leads to detachment (AVI)

Attachment is required for division (AVI)

The authors declare no competing financial interest.

dissemination. Our data suggest that glycocalyx-mediated survival is largely independent of receptor tyrosine kinase and mitogen activated kinase signaling. While anchorage is still required for proliferation, we find that cells with a thick glycocalyx can dynamically attach to a matrix scaffold, undergo cellular division, and quickly disassociate again into a suspended state. Together, our technology provides a needed toolkit for engineering the glycocalyx in glycobiology and cancer research.

Graphical abstract

Keywords

mucin; Muc1; podocalyxin; mechanobiology; glycobiology; synthetic biology

1. INTRODUCTION

The glycocalyx is a polymer meshwork comprising proteins and complex sugar chains called glycans that assemble on the surface of the eukaryotic cell.¹ By mediating receptor–ligand interactions,² cell-to-cell communication,³ and cell-matrix adhesion,⁴ glycans within the glycocalyx are now known to play an essential role in most biological processes, including development,⁵ migration,^{6,7} adhesion,⁸ immune response,^{9–1} and disease progression.^{12,13} A unique feature of the glycocalyx is its dynamic nature. The composition, density, and structural organization of the glycocalyx changes profoundly with cell fate transitions, including differentiation¹⁴ and transformation.^{15,16} A key challenge that remains is to understand on a mechanistic level how changes to the glycocalyx regulate and refine complex cellular and multicellular programs.

While much attention has focused on the biochemical properties of glycans and their chemical interactions, the glycocalyx also functions as an important structural material on the cell surface.^{17–19} Separating the cell membrane and membrane proteins from the local microenvironment, the glycocalyx is uniquely positioned to biophysically regulate cell–extracellular matrix (ECM) and cell–cell interactions.²⁰ For example, the physical properties of the glycocalyx are now known to dictate the kinetics and thermodynamics of integrin ligation with the ECM, thus controlling the spatial organization of integrin bonds and downstream signaling processes.⁸ Moreover, all cell-surface receptors are at least partially embedded within the glycocalyx, and the structural properties of the glycocalyx impact receptor diffusion, activation, and signaling.^{21,22} Consequently, glycocalyx remodeling in

normal physiological processes and disease states is expected to have broad biophysical consequences on cellular signaling and associated behaviors.

Aberrant glycosylation is a universal feature of cancer, but in most cases, the implications of a cancer-specific glycocalyx are poorly understood.^{23,24} For example, circulating tumor cells (CTCs) express an abundance of large, heavily glycosylated proteins, such as the mucin Muc1, which serves as a biomarker for isolation and detection of CTCs in clinical practice.^{8,25,26} Aberrant glycosylation is now recognized to contribute to nearly every step in cancer progression, including proliferation,²⁷ survival,^{7,8,28} angiogenesis,²⁹ and metastatic dissemination.^{30,31} Whether biophysical changes in the glycocalyx also contribute to some of the unique features of aggressive cancer cells and CTCs, including detachment from the ECM and survival in circulation, must still be determined.

A major roadblock to advancing cancer glycobiology has been the relative lack of tools for precision editing of the glycocalyx. One of the most successful approaches to date is based on membrane incorporation of fully synthetic polymers that mimic key features of glycoproteins.^{32,33} Glycopolymers enable tunable control over the types and frequencies of glycoconjugates, membrane densities, and cell surface retention times.^{9,34} The library of glycopolymers, referred to here as the synthetic toolkit, has been applied successfully to investigate cell adhesion processes,⁸ immune cell activation,¹⁰ and host–pathogen interactions.³⁵

Relatively little work has been done in developing a genetic toolbox specifically designed for glycocalyx engineering. A genetically encoded toolbox could provide several unique capabilities that would enable new research in biophysical glycoscience and cancer glycobiology. First, compared to the existing synthetic toolkit, genetic encoding would support prolonged surface expression of glycocalyx elements for long-term *in vitro* and *in vivo* experiments.³⁶ Second, a genetic approach would afford the use of native glycoproteins that are relevant to the particular biological system or disease model of interest. Third, DNA technology provides the flexibility to quickly generate a diverse library of glycoprotein mutants through modern synthetic biology approaches.^{37,38}

Here, we address this unmet need in glycobiology and develop a toolkit of genetically encoded glycoproteins and expression systems to engineer the structure and composition of the cellular glycocalyx. We apply our system to model the CTC glycocalyx and find that the glycocalyx itself could contribute to the unique adhesive properties and survival characteristics of CTCs.

2. RESULTS AND DISCUSSION

System for Stable Incorporation of Engineered Glycoproteins

Our first goal was to develop and validate a strategy for stable expression of glycoproteins in mammalian cells for glycocalyx engineering. We envisioned that incorporation of our constructs and promoters in the cellular genome could (1) provide consistent and reliable levels of glycoprotein expression and glycan presentation, (2) support sorting and selection methods for high expression levels, and (3) enable temporal control over expression through

the use of inducible promoters. Our choice for the promoter was the reverse tetracycline-controlled transactivator (rtTA) system, which can provide temporal control as well as tunable expression levels through titration of doxycycline, the chemical inducer of expression.³⁶ As a test glycoprotein, we chose the mucin Muc1, a key structural element in the glycocalyx of many cancer cell types.^{39,40} For stable integration of the inducible promoter, transgene, and selectable marker, we first tested the utility of standard lentiviral systems (Figure 1A). We found that Muc1 expression levels in epithelial cells were low, and the glycoprotein product was often of lower molecular weight than expected (Figure 1B). We suspected that the highly repetitive sequences in the Muc1 tandem repeats were recombined at some stage of viral packaging or cellular transduction, and we discontinued the further use of lentiviral systems.

We next tested the viability of a transposon-based system^{41–43} for stable expression of large, repetitive glycoproteins like mucins (Figure 1A).⁴¹ We found that Muc1 expression levels were dramatically improved with the transposon system compared to lentiviral transduction (Figure 1B,C). The mucins expressed in transposon-edited cells were heavily glycosylated and had a high molecular weight (Figure 1B). Finally, we confirmed that the mucin expression levels could be tuned through doxycycline induction (Figure 1D,E). On the basis of this performance, the inducible transposon system was applied for all subsequent editing of the glycocalyx.

Genetically Encoded Toolkit for Editing the O-Linked Glycocalyx

Our next goal was to design and fabricate a series of constructs for engineering the structure and *O*-glycan composition of the glycocalyx. Mucin glycoproteins are defined by their densely clustered *O*-glycans, which help to extend and rigidify the mucin polypeptide backbone.^{40,44} As a consequence, mucins extend above most other glycoproteins on the cell surface and are ideal candidates for glycocalyx engineering. Our strategy was to create a library of mutant and semisynthetic mucins that would serve as structural elements and glycan scaffolds on the cell surface. Starting with two relevant mucins in the cancer cell glycocalyx, Muc1 and podocalyxin (Podxl, a structurally analogous glycoprotein⁴⁵), we removed their cytoplasmic signaling domains (CT; Figure 2A,B). Our goal was to minimize the biochemical activity of the mucins so that the mutants would function primarily as structural elements in the glycocalyx. In order to track phenotypic and morphological changes as a function of glycocalyx thickening, we also inserted a fluorescent protein between the *O*-glycan-rich tandem repeats and membrane proximal domains of Muc1 (CT-moxGFP; Figure 2A). To effectively eliminate signaling motifs that could be present in native mucins, we next created a series of semisynthetic constructs. We fused the *O*-glycan rich domains from Muc1 and Podxl to a partially synthetic membrane proximal region, cytoplasmic domain, and transmembrane domain⁴⁶ (SynMuc1 and SynPodxl; Figure 2A,B). We designated these semisynthetic glycoproteins as SynMucins. All constructs were expressed efficiently through our transposon based system and modified the *O*-glycan content of the cell surface accordingly (Figure 2C,D).

We also developed a modular strategy for generating SynMucins of varying length. We introduced Bsu36I restrictions sites that would serve as handles for the removal or addition

of *O*-glycan-rich blocks in our SynPodxl construct. We validated this approach by generating synthetic mucins with O-linked glycosylation domains of 4/3 and 2/3 the length of native Podxl (Figure 3A). In principle, this approach could be adopted to generate constructs of increasingly large size. Lectin blots confirmed the expression of O-rich glycoproteins of varying size by our 2/3, 1/1, and 4/3 length SynPodxl constructs (Figure 3B). Notably, lectin binding to the 2/3 mutant is decreased compared to 1/1 and 4/3 lengths indicating an expected decrease in O-glycosylation due to the decrease in glycosylation sites on the protein. Immunoblot analysis with an anti-Podxl antibody verified expression of the higher molecular weight 4/3 SynPodxl, while the 2/3 length glycoprotein was not detected by immunoblot, likely due to the inadvertent deletion of the antibody recognition motif in this construct (Supporting Figure 1). Together, our library of constructs and expression systems provide a toolkit for expression of structural glycoproteins of varying size and tunable density on the cell surface.

Altering the Chemical and Physical Environment at the Cell Surface

We next tested the ability of our toolkit to modify the chemical composition and physical structure of the cell surface. Our model cell line was the nontransformed mammary epithelial cell (MEC) line, MCF10A, which has low endogenous Muc1 and undetectable Podxl expression. We found that the MCF10A cell line has low overall levels of cell-surface *O*-glycans, making it an ideal system for directed assembly of an *O*-glycan rich glycocalyx (Figure 4A,B). We first demonstrated an ability of our mutant and semisynthetic mucins to alter the chemical environment of the cell surface (Figure 4A,B). We observed significantly increased levels of *O*-glycans on the surface of cells expressing our mutant and semisynthetic mucins compared to those of control MCF10A and Muc1-knockdown cells (Muc1 shRNA). We did not detect changes in N-glycosylation or sialic acids by our mucin constructs as detected by lectins (Figure 4B).

We also tested the performance of our genetic tools in modifying the physical structure of the cellular glycocalyx. We used scanning angle interference microscopy (SAIM),⁴⁷ a fluorescence localization technique with 5–10 nm axial precision, to map changes in the plasma membrane topography and glycocalyx thickness following the expression of our mutant and synthetic constructs (Figure 4C,D). We made all measurements on cells that were adhered to silicon substrates adsorbed with fibronectin. Muc1 knockdown MECs had an average ventral membrane height of 30.3 nm (SD 7.83), while Muc1 CT expression increased the separation between the ventral plasma membrane and the substrate by an average of 120 nm (150.3 nm, SD 15.9) in comparison. The shorter Podxl CT generated a more modest average change of 87 nm (117.3 nm, SD 13.1) compared to the knockdown cells, as expected based on the lower molecular weight of Podxl versus Muc1. Notably, the SynMuc1 and SynPodxl constructs significantly expanded the glycocalyx and performed comparably to the CT mutants of the same glycoproteins (156.6 nm, SD 6.12 and 119.6 nm, SD 12.9, respectively). Together, these results confirm the utility of our toolkit for editing both the chemical composition and physical structure of the glycocalyx.

Thick and Dense Glycocalyx Can Trigger Cellular Detachment from the Matrix

Using our toolkit, we generated cellular models to investigate the consequence of a thick, *O*-glycan rich glycocalyx on cellular behaviors. This glycocalyx is typical of many cancer cells, including highly aggressive cancers presenting with CTCs.⁸ We first tested the implications of dense and thick glycocalyx on cellular adhesion to the ECM. Our analysis focused on our Muc1 mutant construct, which is the largest of our engineered glycoproteins. Notably, we found that a thick and dense glycocalyx could trigger complete cellular detachment from the ECM (Figure 5A,B and Supporting Movie 1). In live-cell time-course experiments that tracked morphological changes dynamically following doxycycline induction, we observed a transition from a classical cuboidal cell phenotype to a more rounded morphology, and finally to a completely detached state, where cells float as spheres above the ECM substrate (Figure 5B and Supporting Movie 1).

The fraction of detached cells correlated with Muc1 surface expression, strongly suggesting that detachment was a consequence of high mucin surface densities (Figure 5C). Indeed, the kinetics of cellular detachment during the first 24 h after doxycycline addition roughly matched the temporal increase in mucin protein expression levels (Figure 5D). We continued to see an increase in the detached cell fraction after 24 h, the time at which maximal protein expression was obtained (Figure 5D). Unexpectedly, we discovered that the majority of the detached cells were viable 24 and 48 h after induction (Figure 5E). On the basis of these observations, we hypothesized that the thick, *O*-glycan-rich glycocalyx enhanced cell survival in poorly adhesive conditions.

Glycocalyx Prolongs Cellular Survival in Suspension

Given the surprising ability of cells with a prominent glycocalyx to survive in anchorage-free conditions, we investigated the survival and proliferation of these cells in suspension culture over longer timeframes. We envisioned these studies would provide a basic model of CTCs, which frequently have a thick *O*-glycan rich glycocalyx and survive, suspended in circulation during dissemination.⁸ We observed that MECs engineered to have a thick and dense glycocalyx live approximately four times longer in suspension than control cells (Figure 6A). The viability of control and engineered cells in suspension was well modeled by an exponential decay with a single decay constant, the half-life. We found that the half-life for MECs with an engineered glycocalyx is 3.12 days (1.96 to 6.51, 95% CI) compared to that of control MECs, which have a half-life of 0.797 days (0.623 to 1.03, 95% CI). We next tested whether this survival phenotype could be attributed to increased receptor tyrosine kinase (RTK) signaling. In a screen of 28 RTKs and 11 signaling nodes, no apparent differences in phosphorylation were observed when comparing engineered and control MECs grown in suspension for 24 h (Figure 6B and Supporting Figure 2). Furthermore, inhibition of MAPK signaling with the inhibitor U-0126 did not significantly change the decay constant for survival in engineered MECs, suggesting the promotion of survival by the glycocalyx was independent of MAPK (Figure 6C).

We next assayed whether cells in suspension were actively proliferating. Despite their prolonged survival in suspension, we observed no proliferation by our engineered cells while in this state indicating that these cells still require anchorage to undergo cell division (Figure

6D). Using time-lapse imaging and single-cell tracking, we determined that the engineered cells proliferate while in an anchored state, but frequently detach from the matrix following division (Figure 6E). Prior to division, cells expressing Muc1-CT adopted a nearly spherical morphology but did not fully detach from the substrate. At the moment of division, however, one or both daughter cells would often lose attachment. Taken together, these results indicate that high levels of cell-surface mucins can trigger a switch from an adhesion-dependent phenotype to an adhesion-free phenotype with enhanced viability.

Cells with a Thick Glycocalyx Dynamically Attach to the Matrix for Proliferation

We next considered two possible mechanisms for how cells with a thick glycocalyx might proliferate: (1) that upon division, the glycocalyx biomass is asymmetrically transferred to daughter cells, leading to a split population of adherent and detached cells or (2) that previously detached cells regain attachment to divide. To test, we generated two populations of Muc1-CT expressing MECs, one with constitutive cytoplasmic EGFP and a second with cytoplasmic mCherry. Twenty-four hours following the induction of Muc1-CT expression, the supernatant of each culture containing detached cells was exchanged, giving mixed cultures of mCherry and EGFP cells, allowing us to track the progress of both starting conditions in a single experiment.

In these experiments, a variety of behaviors were observed, including reattachment of floating cells, detachment of daughter cells following division, and reattachment and spreading of daughter cells following division (Figure 7A and Supporting Movies 2 and 3). Another frequent occurrence was the temporary detachment of one or both daughter cells following division (Figure 7A, row 3). Most interestingly, floating cells also were observed to temporarily attach to the substrate, divide, then detach again (Figure 7A, row 4 and Supporting Movie 4). In the case of the initially attached condition, we observed a monotonically increasing ratio of floating to adherent cell populations over the 28 h following exchange, suggesting that the dynamically adherent subpopulation continuously generated suspended daughter cells (Figure 7B). On the other hand, the initially detached population reached an equilibrium within 10 h of exchange as demonstrated by the constant population ratio (Figure 7C). Similar results were observed for both starting conditions in the mCherry and EGFP cell lines leading to mixed populations in both states, indicating that the transfer was bidirectional for either starting condition (Figure 7D). These results show that cells switch between the substrate attached and free floating states with individual cells occupying both as a function of cell cycle.

3. CONCLUSION

We developed a library of mutant and semisynthetic constructs for expressing heavily O-glycosylated proteins of varying size. Combined with a system for temporal control over expression levels, our systems constitute a genetically encoded toolbox for engineering the cellular glycocalyx. We anticipate that our technology for glycocalyx editing will open new avenues of research previously inaccessible, including more precise modeling of the cancer-specific glycocalyx for *in vitro* and *in vivo* studies. Indeed, the temporal control and stable expression that our systems afford enable dynamic tuning of the glycocalyx in organoids, as

well as syngeneic and xenogeneic mouse models. Furthermore, our system provides new “knobs” for tuning key features of the glycocalyx, including glycan content, glycoprotein size distribution, and glycoprotein surface density.

An important contribution by this work is the design and validation of a series of semisynthetic mucins. While cell-surface mucins have a dominant structural function in the cancer cell glycocalyx, they also have strong biochemical activities mediated by their cytoplasmic and membrane proximal domains.⁴⁸ Our semisynthetic mucins, SynMuc1 and SynPodxl, should enable more reliable physical editing of the glycocalyx, while minimizing the direct biochemical activity of the expressed glycoproteins. We show that our approach for manufacturing synthetic glycoproteins is effective in modulating the thickness and biochemical properties of the glycocalyx. Future studies could add control over the flexural rigidity of individual structural elements. For example, the degree of glycosylation can control the persistence length of mucin biomimetic polymers,⁴⁴ and an analogous strategy to engineer the number of serine and threonine glycosylation sites in genetically encoded mucins could be conducted. As another example, membrane-proximal regions of native Muc1 have been shown to regulate Muc1 trafficking and glycosylation, including extent of glycosylation and sialylation.^{49–51} The dependence of glycosylation on trafficking may explain why we observe more under-glycosylated mucin with SynMuc1, which lacks native membrane proximal and transmembrane domains (Figure 2C). This may also explain the surprising result that, while we increase the *O*-glycan content of the glycocalyx upon expression of our various constructs, we do not observe a significant change in sialic acid content of the glycocalyx (Figure 4B). Additional rounds of protein engineering could also mechanically optimize the hinge point between the *O*-rich glycodomains and our synthetic membrane proximal region to reinforce the glycocalyx under compressive forces. Thus, we envision that future efforts could build on our current library to provide greater control over the physical structure and deformability of the glycocalyx.

Our toolkit overcomes some of the key technical hurdles that have historically limited the generation of cell lines with a cancer-specific glycocalyx. Foremost, we provide a strategy for generating cells with a thick and dense glycocalyx, similar to those of aggressive cancer cells. On the basis of our observation that cells with a thick and dense glycocalyx frequently detach from the ECM substrate, we envision that routine cell culture could quickly select against cells with a prominent glycocalyx. Indeed, many of the existing cancer cell lines have been generated through repeated subculturing, which would likely select against a thick and less compliant glycocalyx that causes cells to detach from the culture vessel. Our inducible system solves this problem by enabling temporal control over glycocalyx biosynthesis in real-time at the start of experimentation.

An unexpected observation in our work is the strong induction of survival by the glycocalyx for cells in a suspended state. Previous work has shown that a thick glycocalyx can support cell survival of anchored cells by promoting integrin assembly, signaling, and cross-talk with growth factor receptor pathways. Now we show that the glycocalyx can also support survival of suspended cells. Importantly, our RTK screen and MAPK inhibitor data suggest that this survival mechanism is independent of growth factor receptor signaling. The precise

mechanism of action for the glycocalyx in this adhesion-independent survival pathway must be resolved in future studies.

In summary, our work describes a set of genetic tools for glycocalyx engineering and rational construction of a cancer-specific glycocalyx in cell lines of interest. Our toolkit provides a necessary technology for addressing unresolved questions in cancer glycobiology, including why CTCs and aggressive cancer cells frequently overexpress large glycoproteins.

4. MATERIALS AND METHODS

Antibodies and Reagents

The following antibodies were used: FITC-human CD227 (Muc1) (559774, BD Biosciences), human CD227 (555925, BD Biosciences) (Muc1), Alexa-488-human podocalyxin (222328, R&D Systems), actin (sc1615, Santa Cruz), goat anti-mouse IgG-HRP (sc-2005, Santa Cruz), and mouse anti-goat IgG-HRP (sc-2354, Santa Cruz). Lectins used were: biotinylated peanut agglutinin (PNA; B-1075, Vector Laboratories), CF568 *Arachis hypogaea* Lectin PNA (29061, Biotium), CF640R *Arachis hypogaea* lectin PNA (29063, Biotium), CF633 wheat germ agglutinin (WGA; 29024, Biotium), and FITC concanavalin A (ConA; FL-1001, Vector Laboratories). Biotinylated lectins were detected using ExtrAvidin-Peroxidase (E2886, Sigma). MAPK inhibitor was U-0126 (70970, Cayman Chemical). To induce transactivator cell lines, doxycycline was used (sc-204734, Santa Cruz).

Cloning and Constructs

Full details on cloning and assembly of lentiviral, piggybac, and transient expression vectors with SynMucins are given in the Supporting Information.

Cell Lines and Culture

MCF10A and HEK293T cells were obtained from ATCC. MCF10A cells were cultured in DMEM/F12 media supplemented with 5% horse serum, 20 ng/mL EGF, 10 µg/mL insulin, 500 ng/mL hydrocortisone, and 100 ng/mL cholera toxin. HEK293T cells were cultured in DMEM high glucose supplemented with 10% fetal bovine serum. Cells were maintained at 37 °C, 5% CO₂, and 90% RH. MCF10A shRNA Muc1 cells were prepared by lentiviral transformation using Muc1 shRNA pLKO.1. MCF10A rtTA stable line was prepared by lentiviral transformation using pLV rtTA-NeoR plasmid as previously described.^{8,47} Cells were further modified with the transposon system to create stable lines expressing Muc1 CT; SynMuc1; Muc1 CT moxGFP; Podxl CT; and 2/3, 1/1, and 4/3 SynPodxl. These stable lines were generated using Nucleofection Kit V (Lonza) and HyPBBase, an expression plasmid for a hyperactive version of the PiggyBac transposase⁵² (kindly provided by Dr. Alan Bradley, Wellcome Trust Sanger Institute, UK). MCF10A rtTA Muc1 CT and MCF10A rtTA SynMuc1 were sorted for expression using the FITC-Human CD227 antibody. MCF10A rtTA Muc1 CT EGFP and MCF10A rtTA Muc1 CT mCherry cells were generated by further lentiviral modification of MCF10A rtTA Muc1 CT cells with pLenti EGFP (Addgene plasmid #17446⁵³) and pCDH mCherry, respectively. Additionally, a Muc1 CT stable line was created by lentiviral transformation of the MCF10A rtTA cell

line using a pLV tetOn Muc1 CT plasmid. HEK293T cells were transiently transfected using a calcium phosphate transfection protocol with pMAX GFP, pcDNA3.1-(+) 2/3 SynPodxl, pcDNA3.1(+) 1/1 SynPodxl, and pcDNA3.1(+) 4/3 SynPodxl plasmids.

Immunoblot Analysis

Cells were plated at 20,000 cells/cm² and grown for 24 h. Cells were then induced with 0.2 µg/mL doxycycline for 24 h before lysis with Tris-Triton lysis buffer (Abcam). Any detached cells were included in lysates. Lysates were separated on NuPage 4–12% Bis-Tris gels and transferred to PVDF membranes. Membranes were blocked with 3% BSA TBST solutions for 2 h at room temperature or overnight at 4 °C. Primary antibodies were diluted 1:1000 and lectins were diluted to 1 µg/mL in 3% BSA TBST and incubated 4 h at room temperature or overnight at 4 °C. Secondary antibodies or ExtrAvidin were diluted 1:2000 in 3% BSA TBST and incubated for 2 h at room temperature. Blots were developed in Clarity ECL (BioRad) substrate, imaged on a ChemiDoc (BioRad) documentation system, and quantified in Fiji.⁵⁴

Flow Cytometry

Cells were plated at 20,000 cells/cm² and grown for 24 h. Cells were then induced with 0.2 µg/mL doxycycline for 24 h. Adherent cells were nonenzymatically detached by incubating with 1 mM EGTA in PBS at 37 °C for 20 min and added to the population of floating cells, if present. Antibodies were diluted 1:200, and lectins were diluted to 1 µg/mL in 0.5% BSA PBS and incubated with cells at 4 °C for 30 min. The BD Accuri C6 flow cytometer was used for analysis. For doxycycline-titration flow cytometry, Muc1 CT moxGFP expressing cells were plated and induced similarly with varying concentrations of doxycycline (0, 1, 5, 10, 25, 50, 75, 100, 200, 350, 500, and 1000 ng/mL). Cells were nonenzymatically detached as described above and fixed with 4% paraformaldehyde, and the GFP signal was analyzed on the BD Accuri C6 flow cytometer. Median GFP signal is reported.

Confocal Microscopy

Cells were plated at 5,000 cells/cm² and grown for 24 h and subsequently induced with 0.2 µg/mL of doxycycline for 24 h before being fixed with 4% paraformaldehyde. Samples were blocked with 5% normal goat serum PBS for 1 h at room temperature. Antibodies were diluted 1:200 in 5% normal goat serum PBS and incubated overnight at 4 °C. Lectins were diluted to 1 µg/mL in 5% normal goat serum PBS and incubated for 2 h at room temperature. Samples were imaged on a Zeiss LSM inverted 880 confocal microscope using a 40× water immersion objective.

Cell Detachment Time Course

Cells were plated at 20,000 cells/cm² and grown for 24 h. All media were changed to remove any detached cells at this time. Cells were induced by spiking 0.2 µg/mL doxycycline into the media at the designated time points. Detached cells were collected, stained with Sytox-Green (Thermo), and analyzed with a BD Accuri C6 flow cytometer.

Suspension Cultures

Cells were grown and induced with 0.2 $\mu\text{g/mL}$ doxycycline for 24 h using an adherent cell culture technique. Cells were then detached with 0.05% trypsin EDTA (Thermo) and resuspended at 375,000 cells/mL. Cells were grown in suspension using a cell-culture magnetic stirrer at 100 rpm, 37 °C, 5% CO₂, and 90% RH. Every 24 h, a small sample was drawn, and cells were counted with a hemocytometer using Trypan Blue (Thermo) to differentiate live and dead cells. For inhibitor studies, 10 μM MAPK inhibitor or DMSO (control) was added when cells were transferred to suspension.

RTK Screen

Cells were grown in suspension or adherent populations and induced for 24 h. Cells were then lysed per the previous discussion with the addition of sodium fluoride and sodium orthovanadate to the lysis buffer. The PathScan RTK Signaling Antibody Array Kit (7982, Cell Signaling) protocol was used to complete the assay using 0.5 mg/mL of each lysate.

EdU Proliferation Assay

Click-iT Plus EdU Alexa Fluor 594 Flow Cytometry Assay Kit (Thermo) was used. Cells were grown in suspension for 22 h as described above before adding 10 μM EdU to the culture for 2 h. Adherent cells were plated at 20,000 cells/cm² and grown for 46 h before adding 10 μM EdU to the culture. The procedure was completed per the assay kit manual, and samples were analyzed on a BD Accuri C6 flow cytometer.

Scanning Angle Interference Microscopy

Silicon wafers with 1900 nm thermal oxide were purchased from Addison Engineering and diced into 1 cm² chips, cleaned in 5 M NaOH, rinsed extensively with deionized water, then oxygen plasma cleaned for 60 s before coating with 50 $\mu\text{g/mL}$ human plasma fibronectin (Millipore) for 2 h at 37 °C. Cells were seeded onto the prepared substrates at 5,000 cells/cm² and cultured for 24 h before the addition of doxycycline, cultured for an additional 24 h, then fixed in 4% paraformaldehyde at 37 °C for 15 min. Following fixation samples were rinsed extensively in PBS, then stained with 1 $\mu\text{g/mL}$ DiI-DS (ThermoFisher) for 1 h at RT. Samples were imaged with a custom SAIM microscope based on a Nikon Ti-E body with ultrastage and focus lock (FocalPoint) with a 560 nm excitation laser (MFB). Experiments were automated with a homemade microscope controller and galvanometer scanning mirror (Cambridge Technology), and images were acquired over a series of 62 angles from 0 to 36.7° with a 60 $\times$ NA 1.2 water immersion objective (Nikon) on a sCMOS camera (Zyla 4.2, Andor) controlled with μ Manager software (Open Imaging).⁵⁵ Image sequences were fit pixel-wise in a least-squares sense according to the SAIM optical model⁴⁷ with a custom software application (source code available upon request), and the resulting reconstructions were postprocessed using Fiji. For each condition, 2–4 independent samples were imaged. Because of variability in label density and the effects of dye aggregates in many cells, only a small portion of the membrane provided an adequate reconstruction. Cells with globally low residuals and continuous 40 $\times$ 50 (2k total) pixel regions in the reconstructions were selected from the pooled samples after reconstruction. A rectangular box of 40 $\times$ 50 pixels was drawn directly under the cell body, as the region on

the periphery is prone to artifacts in reconstruction owing to the membrane being oriented roughly parallel to the optical axis or the close proximity between the ventral and apical membranes. The pixels heights within these boxes were used for quantification of membrane height.

In-incubator Microscope

Live cell time-course imaging was carried out on a custom-built microscope designed to operate in a standard cell culture incubator. The microscope consists of a mechanical XY stage, with separate focus drive, and filter wheel controlled by a central hub (ASI Tiger) and operated through μ Manager control software.⁵⁵ Excitation by LED sources (ThorLabs) was controlled by an Arduino board through μ Manager. A Nikon 10 $\times$ NA 0.50 CFI S Fluor objective was used to image onto a CMOS camera (PointGrey). Full details on microscope design are available upon request.

Live-Cell Time-Course Imaging

Glass bottomed cell culture plates (Cellvis) were oxygen plasma cleaned for 60 s then coated with 50 μ g/mL human plasma fibronectin for 2 h at 37 °C. Cells were seeded at 5,000 cells/cm² and cultured for 24 h prior to the addition of doxycycline. Following induction, the sample was placed on the in-incubator microscope and allowed to equilibrate for 4 h. Images were then acquired for 20 h at multiple positions in 2 different wells for induction time-course series. For adhesion/detachment studies, cells were initially imaged for 24 h following induction, then the plate removed and supernatant swapped between each of 3 wells of EGFP and mCherry cell lines. The plate was then replaced and imaging continued for an additional 33 h. Images were analyzed with custom analysis software written by the authors and available upon request. Briefly, in each frame circular cells and all cells are identified, then a mean intensity calculated for all cells in the frame. The number of adherent cells is then inferred as the difference between circular and total cells.

Statistics

Statistical significance was determined by Student's *t* test, two-tailed, or ordinary one-way ANOVA as appropriate. Statistical analysis was performed using Prism (GraphPad).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Vector for stable expression. (A) Graphic illustration of the lentiviral and transposon stable incorporation systems. (B) Representative immunoblot (left) and lectin blot (right) comparison of stable Muc1 expression and PNA binding in lentiviral infection versus transposon integration, $n = 3$. (C) Mean integrated signal density from α -Muc1 immunoblots in B normalized to lentiviral samples; error bars represent the SD, $n = 3$. (D) Immunoblot (left) and lectin blot (right) of Muc1 expression in w.t. MCF10A cells compared to stable expression lines uninduced and after 24 h induction with $0.2 \mu\text{g mL}^{-1}$ doxycycline, $n = 1$.

Cell lines were prepared with the transposon incorporation system. (E) Fold change in Muc1 analyzed by flow cytometry upon induction with various doxycycline concentrations, $n = 3$. * $p < 0.05$; ** $p < 0.01$ (two-tailed t test).

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Figure 2.

Genetically encoded glycoproteins for glycocalyx editing. (A) Schematic representation of the components and features of the native and engineered glycoproteins used in this study. S, signal sequence; VNTR, variable number tandem repeat; SEA, sea-urchin sperm protein, enterokinase, and agrin domain; TM, transmembrane domain; CT, cytoplasmic tail; FP, fluorescent protein; TM21, synthetic transmembrane domain, 21 amino acids; MP, membrane proximal domain; and ED, O-glycosylation rich ecto-domain region. (B) Cartoon illustration of the approximate relative size and features of various engineered glycoproteins. (C) Immunoblot (left) and lectin blot (center) showing the molecular weights and expression levels of Muc1 mutants in mammary epithelial cells (MECs) stably expressing the indicated gene, $n = 3$. Flow cytometry histograms (right) of the α -Muc1 antibody binding in cells expressing each of the engineered mutants compared to knockdown (shRNA) and w.t. cells, $> 50,000$ cells measured per condition. (D) Immunoblot (left) showing the relative size and expression level of Podxl mutants in stable MEC cell lines, $n = 3$. Flow cytometry

histograms (right) of α -Podxl antibody binding in the same cell lines; >50,000 cells measured per condition.

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Figure 3.

Glycoproteins of tunable length. (A) Schematic illustration of the restriction sites introduced via mutation (*), reassembly fragments, and relative lengths of the engineered SynPodxl variants. (B) Lectin blot (left) of the relative O-glycosylation level of each mutant transiently expressed in HEK293T cells and an increased exposure time of the same lectin blot (right), $n = 2$.

Figure 4.

Engineering the chemical and physical properties of the glycocalyx. (A) Confocal images of Muc1 (left), O-glycan (center left), and sialic acid (center right) showing cell surface localization in control and stably expressing MECs for each indicated construct (scale bar 25 μ m), $n = 3$. (B) Representative flow cytometry histograms showing cell surface O and N-glycan and sialic acid levels of Muc1 and Podxl CT and SynMucin mutants; >20,000 cells measured per condition and $n = 3$. (C) Representative fluorescence maximum intensity projections of SAIM image sequences (top left, second row, scale bar 10 μ m) and membrane

height reconstructions (top right, scale bar 10 μm , bottom row, scale bar 5 μm) of the ROIs boxed in red. (D) Average membrane height from 2k pixels in each of 5 cells per condition. Boxes correspond to SD and whiskers to extrema. Asterisks indicate statistical comparison of each mucin-expressing cell line to shRNA Muc1 expressing cells. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (one-way ANOVA).

Figure 5.

Expression of a bulky glyocalyx inhibits cellular adhesion. (A) Representative phase-contrast images of MECs expressing Muc1-CT, uninduced, and induced for 24 h (scale bar 100 μm). (B) Representative epifluorescence images of MECs expressing Muc1-CT-moxGFP 4, 12, and 20 h postinduction (scale bar 100 μm), $n = 2$. (C) Ratio of detached MECs to adherent as a function of relative Muc1-CT-moxGFP fluorescence intensity, $n = 2$. (D) Concentration of live, detached MECs expressing Muc1-CT as a function of time postinduction, quantified by flow cytometry with live/dead cell stain, $n = 3$. (E) Percentage of live cells in total detached population at various time points after induction quantified by flow cytometry with live/dead cell stain, $n = 3$. All error bars are SD.

Figure 6.

Expression of a bulky glycocalyx enhances cell survival in suspension. (A) Percentage live cells as a function of time for MECs growing in suspension culture, $n = 3$. The solid curves represent a single exponential decay fit, $t_{w.t.}^{1/2} = 0.797d$, $t_{Muc1}^{1/2} = 3.12d$. (B) Receptor tyrosine kinase signaling screen for MECs grown in suspension in full serum for 24 h, $n = 2$. Visible signal from positive controls. See Supporting Figure 2 for full details. (C) Percent live MECs expressing Muc1 Δ CT grown in suspension with 10 μ M MAPK inhibitor U-0126 or DMSO control, $n = 2$. (D) Representative flow cytometry histogram showing incorporation of EdU

into MECs under adherent or in suspension culture conditions; > 100,000 cells measured per condition, $n = 2$. Percentage indicates the fraction of cells with signal $> 10^4$ (gray line) after background subtraction. (E) Epifluorescence images of an MEC undergoing division with increasing expressing of Muc1-CT-moxGFP. Following separation, one of the new cells retains attachment to the substrate (white arrow), while the other detaches and drifts out of frame (red arrow) (scale bar 50 μm). All error bars are SD. Timestamp shown in hour:min.

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Figure 7.

Cellular division is associated with adhesion. (A) Live cell time-course images of a detached cell reattaching to the substrate (top), a division event wherein one of the new cells detaches from the substrate (top center, white arrow), a division event wherein a new cell briefly detaches then reattaches to the substrate (bottom center, white arrow), and a detached cell attaches to the substrate, divides, then one of the new cells detaches (bottom, white arrow) (scale bars 50 μ m). (B) Ratio of detached, floating cells to adherent as a function of time in an initially attached population expressing Muc1 CT. (C) The same measurement as B for an initially detached population. Error bars in B and C represent one SD, $n = 3$ for both conditions. (D) Epifluorescence image of a mixed population of MECs expressing Muc1 CT and either cytoplasmic EGFP (green, initially detached) or mCherry (magenta, initially attached) demonstrating the exchange of states occurs in both directions (scale bar 100 μ m).