

STATEMENT OF PURPOSE

Study Rationale

Physical cues of the cellular microenvironment are fundamental for normal physiology, and yet little is understood regarding how these factors influence the cellular responses. Pivotal studies have begun uncovering the importance of how these cues govern cell fate and behavior. Engler et al. first demonstrated that substrate physical stiffness can dictate mesenchymal stem cell (MSC) lineage (1). Shortly after, Yes-associated protein (YAP) and its paralog transcriptional coactivator with PDZ-binding motif (TAZ) have been discovered as central molecular transducers of extracellular mechanical forces (2). YAP/TAZ mechanosignaling has since been implicated as potent regulators of organogenesis, tissue regeneration, and cancer progression (reviewed in refs (3, 4)).

A recent study by Gouveia et al. demonstrated that human limbal epithelial stem cells (LESCs), that are fundamental for cornea epithelial turnover, reside in considerably softer pockets of the limbus where stiffness of the microenvironment is reduced and YAP-dependent mechanotransduction is inhibited. They induced alkali burns into rabbit corneas and demonstrated that stiffening of the cornea was correlated with a loss of putative limbal stem cell markers. They next showed that collagenase treatment of the injured corneas reduced its stiffness and, remarkably, prompted a return of limbal stem cell markers (5).

These pioneering studies necessitate a deeper look into understanding the molecular transducers of mechanical forces. To study mechanobiology, scaffolds with tunable physical properties are required. Previous work from our lab has demonstrated that films cast from regenerated silk fibers of the *Bombyx mori* silkworm is a biomaterial uniquely suited for corneal therapy (6-14). These films are optically transparent, mechanically strong, non-immunogenic, and support corneal cell growth. The physical properties of these films are also highly tunable and offer an excellent tool to regulate substrate stiffness and study corneal cell biomechanics. This study will use silk films as a tool to examine the effects of substrate stiffness on corneal epithelial stemness.

Hypothesis

We hypothesize that soft silk films (with reduce stiffness) are able to upregulate limbal stem cell markers through inhibition of YAP1 nuclear signaling. Of particular interest is the putative stem cell marker SOX9 (15, 16). YAP1 associates with transcriptional factor TEAD and can subsequently bind to the promoter region of the *SOX9* gene (17). We hypothesize that the putative limbal stem cell marker SOX9 is mechanosensitive through its association with YAP1.

Specific Objectives

1. Characterize the expression of limbal stem markers and differentiation markers in response to silk stiffness
2. Characterize the limbal epithelial YAP1-SOX9 axis in response to silk stiffness.

Clinical Relevance

While the field of mechanobiology is still in its infancy, both its clinical and scientific implications are extensive. A better understanding of the cellular mechanotransducers will provide crucial insight into an area of cell behavior that has, until now, been largely overlooked (reviewed in ref (18)). This work has direct implications on our understanding of LESCs and corneal wound healing. This opens the possibility of engineering biomaterials that manipulate LESC behavior for the purposes of ocular therapy.

PRELIMINARY DATA

Silk film stiffness can be modified over ten-thousand fold

Methanol immersion of silk films induces physical crosslinking in the form of beta-sheet crystal structures. By modifying the concentration of methanol used, the stiffness of silk films can be changed over ten-thousand fold.

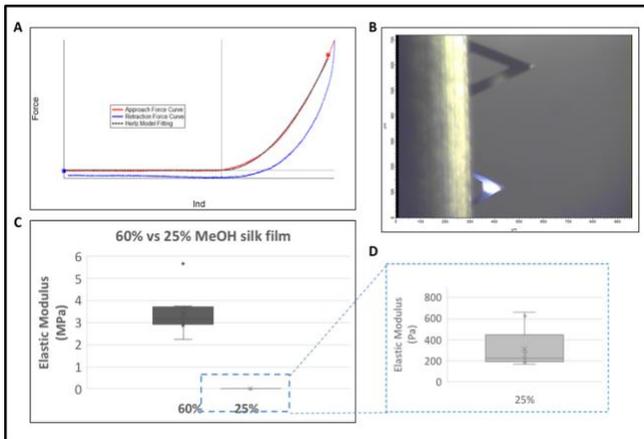


Fig. 1. Stiffness of 25% and 60% methanol immersed silk film was measured using Atomic Force Microscopy (AFM) (A) The Young's modulus (stiffness) was determined by fitting the linear elastic Hertzian contact model to estimate the slope of the stress vs. strain AFM force curves. **(B)** Example AFM cantilever tips used to indent silk film samples. **(C-D)** Young's modulus of 60% methanol films measured $3.40 \times 10^6 \pm 0.96 \times 10^6$ Pa, while modulus of 30% methanol films measured 307.43 ± 193.43 Pa. This is over a ten-thousand fold difference in stiffness.

HCLE cell morphology is significantly altered when grown on soft versus stiff silk films

Cell spreading is used as an indicator of cell response to substrate stiffness and correlates directly with YAP nuclear translocation. Stiff substrates allow for greater stability of focal adhesion and cytoskeletal structures, thereby allowing the cell to spread more along the substrate surface (2).

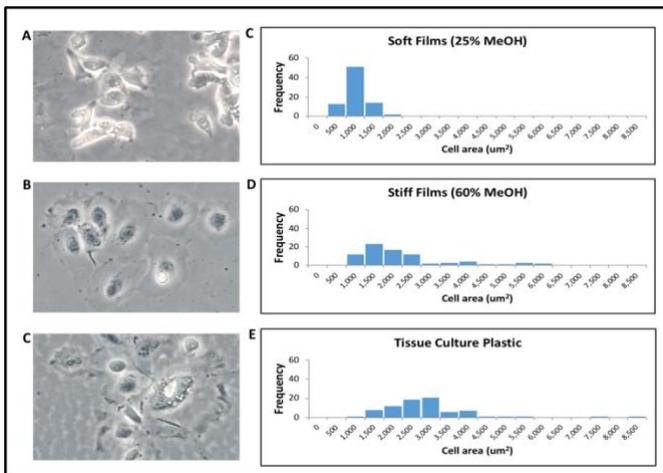


Fig. 2. HCLE cell spreading is inhibited on soft silk films. (A-C) HCLE cells grown on soft (25% methanol) films are morphologically more rounded and smaller compared to cells grown on stiff (60% methanol) films and TCP. **(C-E)** Cell area, measured by manual tracing, revealed that HCLE cells grown on soft films were smaller and more uniform than cells grown on stiff films or TCP.

YAP signaling is inhibited in HCLE cells grown on soft silk films

To corroborate the results observed with HCLE cell morphological changes, these cells on soft films, stiff films, and TCP were immunostained with YAP1 antibodies. Images reveal YAP1 nuclear localization was inhibited on soft silk films, confirming our initial hypothesis.

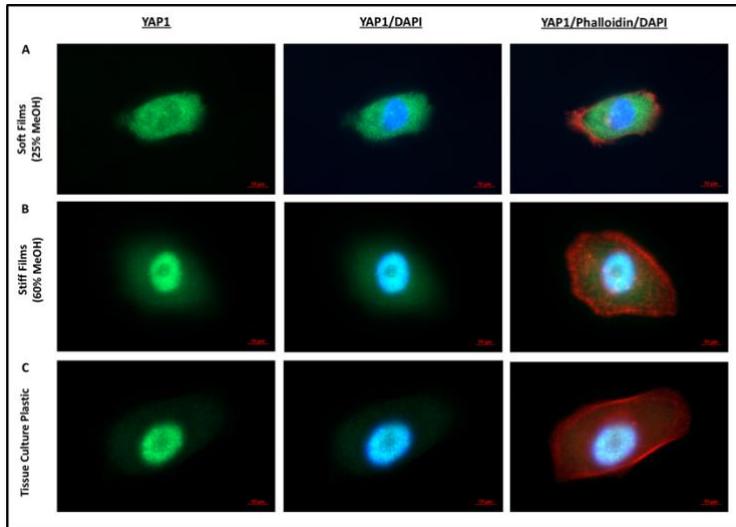


Fig. 3. HCLE cells seeded on different stiffness substrates stained with YAP1, phalloidin, and DAPI. (A) Nuclear localization of YAP1 transcriptional co-activator is inhibited in cells grown on soft films. **(B)** YAP1 is primarily nuclear and co-localizes with DAPI stain in cells grown on stiff films. **(C)** Similar nuclear localization of YAP1 seen in cells grown on TCP. Less cytoplasmic YAP1 observed in TCP cells compared with stiff films.

Actin stress fibers are absent in HCLE cells grown on soft silk films

YAP1 nuclear translocation and subsequent signaling is hypothesized to depend on cytoskeletal tension (2). A common indicator of cytoskeletal tension is the formation of actin stress fibers. Actin staining with phalloidin-conjugated fluorophores revealed absence of stress fibers on soft silk films, suggesting that cytoskeletal tension is correlated with YAP1 signaling

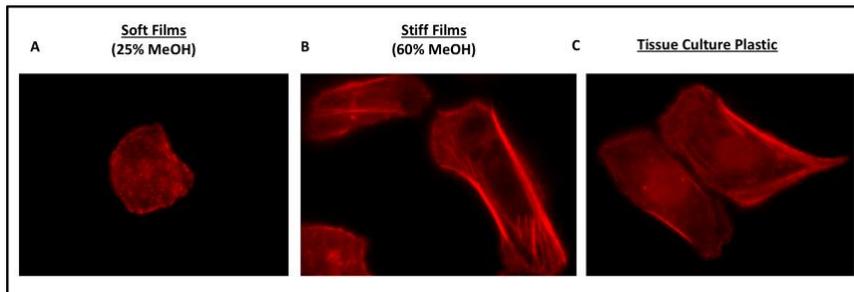


Fig. 4. HCLE cells seeded on different stiffness substrates stained with actin cytoskeletal marker phalloidin. (A) HCLE cells on soft films lacked actin stress fibers and were more spherically shaped. **(B)** Cells on stiff films displayed actin stress fibers and a more elongated morphology. **(C)** Similar stress fibers and cell morphology are seen in cells grown on TCP.

RESEARCH PLAN

Study Design & Methods

Specific Aim 1: Characterize Corneal Limbal Epithelial Cell Response to Silk Film Stiffness.

We hypothesize that limbal stem/progenitor markers will be upregulated on soft silk films and epithelial differentiation markers will be upregulated on stiff films. Concurrently, we believe colony formation efficiency will be improved on soft films. Gouveia et al. found that limbal stem/progenitor markers were upregulated both *in vivo* and *in vitro* on soft matrices where YAP1 signaling was inhibited (5). This aim will characterize expression of limbal stem/progenitor and epithelial differentiation markers in response to silk films stiffness. Characterization will first be done in immortalized human corneal limbal epithelial (HCLE) cells. This will be followed by functional assessment of the colony forming efficiency with primary murine limbal stem/progenitor cells.

Milestone 1.1 Characterize expression of limbal stem/progenitor markers in HCLE cells on soft and stiff films. HCLE cells will be plated on soft (25% methanol) and stiff (60% methanol) silk films at the same density. Cells will be fixed and fluorescently stained with limbal stem/progenitor markers ABCG2, CK15, and $\Delta Np63$. Images will be taken (Zeiss AxioObserver) and analyzed for differential expression between soft and stiff films.

Milestone 1.2 Characterize expression of epithelial differentiation markers in HCLE cells on soft and stiff films. HCLE cells will be plated on soft and stiff silk films at the same density. Cells will be fixed and fluorescently stained with epithelial differentiation markers CK3, IVL, and KRT12. Images will be taken (Zeiss AxioObserver) and analyzed for differential expression between soft and stiff films.

Milestone 1.3 Colony formation assay on soft and stiff silk films with primary murine limbal stem/progenitor cells. Our lab has generated an inducible transgenic “pulse-chase” murine model (K5Tta \times TRE-H2BGFP) to localize, purify, and characterize slow cycling cells in the cornea. We have previously demonstrated this system can label virtually all limbal stem cells in the murine cornea (16). These corneas will be harvested and slow cycling cells will be purified via fluorescence activated cell sorting (FACS). Purified cells will be initially plated on MMC treated NIH-3T3 cells. Colonies will be identified by light microscopy and holoclones will be plated onto soft or stiff silk films. Results will assess the ability of soft and stiff silk films to maintain limbal stem/progenitor phenotype.

Specific Aim 2: Characterize Corneal Epithelial Cell YAP1-SOX9 Axis in Response to Silk Film Stiffness. We hypothesize that putative limbal stem cell marker SOX9 is mechanosensitive through association with YAP1 mechanotransducer. SOX9 has been implicated as a potential limbal stem cell marker (15, 16). Furthermore, not only does the YAP1-TEAD complex bind directly to the promoter region of the SOX9 gene, the YAP-SOX9 axis has been associated with driving stemness and progression of esophageal carcinoma (17). This aim will characterize the YAP1-SOX9 axis in corneal limbal epithelial cells.

Milestone 2.1 Determine expression of YAP1 and SOX9 in HCLE cells grown on soft/stiff films.

HCLE cells will be plated on soft or stiff silk films at the same density. Cells will be fixed and immunostained with YAP1 and SOX9 antibodies. To quantify mRNA and protein levels, qPCR and western blot will be performed.

Milestone 2.2 Inhibit YAP1 binding with TEAD transcriptional factor and determine SOX9 localization in HCLE cells grown on soft/stiff films.

HCLE cells plated on soft and stiff films will be treated with verteporfin to inhibit YAP1-TEAD association. SOX9 will be stained to determine localization, and qPCR and western blot will be performed to quantify mRNA and protein expression, respectively. Results will be compared to HCLE cells plated on silk films without verteporfin treatment.

Milestone 2.3 Determine localization of Sox9 in primary murine limbal stem/progenitor cells grown on soft/stiff films.

Corneas from K5Tta × TRE-H2BGFP mice will be harvested and slow cycling cells will be purified via FACS. Purified cells will be plated on soft and stiff silk films at the same density. Cells will be fixed and immunostained with Sox9 antibodies to determine expression and localization.

Statistical Plan

Statistical analysis will be performed with GraphPad Prism software. Gene expression levels from qPCR will be determined by normalizing to GAPDH and calculating $\Delta\Delta CT$ values. Analysis will be performed using one-way analysis of variance (ANOVA) with a post-hoc Tukey test.

Immunofluorescence will be qualitatively assessed for protein localization. Significance in all cases will be determined by p-value <0.05.

Anticipated Results

Milestone 1.1 Limbal stem/progenitor markers will be upregulated in HCLE cells soft silk films. Cells with upregulated stem/progenitor markers will also display cytoplasmic localization of YAP1.

Milestone 1.2 Epithelial differentiation markers will be upregulated on stiff silk films. Cells with upregulated differentiation markers will also display nuclear localization of YAP1

Milestone 1.3 Colony forming efficiency of primary murine limbal stem/progenitor cells will be improved on soft silk films.

Milestone 2.1 Nuclear localization of YAP1 will correspond to an inhibition of SOX9 protein and mRNA expression in HCLE cells.

Milestone 2.2 Inhibition of YAP1-TEAD interactions by verteporfin will allow for SOX9 expression on stiff silk films

Milestone 2.3 Sox9 protein expression in primary murine limbal stem/progenitor cells will be highly expressed on soft silk films while inhibited on stiff films.

Pitfalls

- Stiffness of silk films may need to be titrated to obtain the appropriate range for corneal limbal cell response. The stiffness of silk films are easily changed by modifying the concentration of methanol. Films will be measured by AFM to determine appropriate Young's modulus.
- The results of the immunofluorescent stains largely depend on the efficacy of antibodies used. I will use antibodies that have been validated in the literature and in previous studies published in our lab.

- Colony formation assay with primary murine limbal stem/progenitor cells may be challenging. Our lab a standardized protocols and have published previous work with K5Tta × TRE-H2BGFP mice (16) and colony formation assays on silk films (6).

Alternative Strategies

Alternative materials can be used to study corneal limbal cell mechanotransduction. Synthetic polyacrylamide (pAA) gels are the standard for studying cell response to substrate stiffness. While less clinically applicable than silk films, pAA gels can be used as an alternative in this project. Silk hydrogels induced through chemical crosslinking, rather than B-sheet physical crosslinks, can also be explored as an alternative technique. These hydrogels are also able to achieve a wide range of stiffnesses and support cell growth and can be used as another alternative.

Timeline

| Research | Project Timeline | | | | | | | | | | | | Benchmarks |
|--|------------------|---|---|---|---|----|---|----|---|----|----|--|---|
| | Jl | A | S | O | N | Ja | F | Mr | A | My | Jn | | |
| SA1: Corneal Limbal Epithelial Cell Response to Silk Film Stiffness | | | | | | | | | | | | | |
| Milestone 1.1 Characterize expression of limbal stem/progenitor markers in HCLE cells on soft and stiff silk films | | | | | | | | | | | | | Determine if limbal markers ABCG2, CK15, nuclear β -catenin, and Δ Np63 are expressed more on soft or stiff silk films |
| Milestone 1.2 Characterize expression of epithelial differentiation markers in HCLE cells on soft and stiff silk films | | | | | | | | | | | | | Determine if epithelial differentiation markers CK3, lvl, and KRT12 are expressed more on soft or stiff silk films |
| Milestone 1.3 Colony formation assay on soft and stiff silk films with primary murine limbal stem/progenitor cells | | | | | | | | | | | | | Determine if colony forming efficiency is altered on soft or stiff silk films |
| SA2: Characterize Corneal Epithelial Cell YAP1-SOX9 Axis in Response to Silk Film Stiffness | | | | | | | | | | | | | |
| Milestone 2.1 Determine expression of YAP1 and SOX9 in HCLE cells grown on soft and stiff films | | | | | | | | | | | | | Determine if YAP1 nuclear localization correlates with SOX9 expression and localization |
| Milestone 2.2 Inhibit YAP1 binding with TEAD transcriptional factor and determine SOX9 expression | | | | | | | | | | | | | Determine if SOX9 expression is affected by inhibition of YAP1-TEAD interaction |
| Milestone 2.3 Determine localization of Sox9 in primary murine limbal stem/progenitor cells grown on soft and stiff films | | | | | | | | | | | | | Determine localization of Sox9 in primary mouse limbal stem/progenitor cells |

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