

# Silk-Derived Protein Enhances Corneal Epithelial Migration, Adhesion, and Proliferation

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**PURPOSE.** The corneal surface is vulnerable to a myriad of traumatic insults including mechanical, chemical, and thermal injuries. The resulting trauma may render the naturally occurring regenerative properties of the cornea incapable of restoring a healthy epithelial surface, and may result in the loss of corneal transparency and vision. Healing of the corneal epithelium requires a complex cascade of biological processes that work to restore the tissue after injury. New therapeutic agents that act on the multiple steps of the corneal wound-healing process would offer a potential for improving patient outcomes. Here, a novel silk fibroin-derived protein (SDP) was studied for potential impacts on wound healing through studying an in vitro model.

**METHODS.** Solubilized SDP, produced from the *Bombyx mori* silkworm cocoon, was added to human corneal limbal-epithelial (hCLE) cultures to evaluate the material's effects on epithelial cell migration, proliferation, and adhesion through the use of various scratch wound assays and flow chamber studies.

**RESULTS.** Results indicated that the addition of SDP to culture increased hCLE migration rate by over 50%, and produced an approximate 60% increase in cell proliferation. This resulted in a nearly 30% enhancement of in vitro scratch wound closure time. In addition, cultures treated with SDP experienced increased cell-matrix focal adhesion formation by over 95% when compared to controls.

**CONCLUSIONS.** The addition of SDP to culture media significantly enhanced hCLE cell sheet migration, proliferation, and attachment when compared to untreated controls, and indicates SDP's potential utility as an ophthalmic therapeutic agent.

**Keywords:** corneal epithelium, cell migration, cell adhesion, corneal wound healing, silk protein

The corneal epithelium is the outermost layer of the cornea and vulnerable to a range of traumatic mechanical, chemical, and thermal insults as well as damage from the use of ocular medications, surgical procedures, and immune processes.<sup>1</sup> Annually, over 50 million people worldwide suffer from blindness as a result of corneal disease with nearly 2.5 million new eye injuries occurring each year in the United States. Altogether, corneal surface injuries represent the single most important ocular environmental health problem in society.<sup>2</sup> Corneal injuries are extremely painful, and severe damage may render the tissue's natural regenerative abilities incapable of restoring a healthy epithelial surface.<sup>3</sup> Corneal wound healing involves a set of highly regulated cell behaviors and an organized cascade of mechanisms that work together in concert to re-epithelialize the corneal surface and restore the functional integrity of the tissue.<sup>4</sup> On the normal ocular surface, corneal epithelial defects are rapidly repaired by the migration of adherent basal epithelial cells to first cover the defect, followed by the proliferation and differentiation of limbal stem cells to reconstitute a nonkeratinized stratified architecture.<sup>5</sup> A stunted healing response may ultimately lead to the occurrence

of corneal disease and a loss of vision from a poorly refracting ocular surface.<sup>6</sup>

The use of eye drops is an established drug delivery method for treating ocular conditions owing to their ease of use by most patients and wide applicability. However, clinical approaches to treating corneal injuries with eye drop formulations have been relatively limited owing to the lack of available effective product options.<sup>7</sup> To date, most therapeutic eye drop formulations are used in clinically reactive approaches (e.g., steroids, antibiotics) to treat acute ophthalmic problems that do not address the wound-healing process through tissue regenerative approaches.<sup>8</sup> The only current regenerative approach to treating ocular surface injury is the use of amniotic membrane, an exogenous tissue derived from the human placenta, to facilitate the healing and restoration of the injured corneal epithelium.<sup>9</sup> However, amnion may have drawbacks due to inconsistent material properties in terms of thickness, degradation rate, mechanical integrity, and biochemical composition.<sup>10</sup> In addition, the application of amnion to the ocular surface is surgically invasive and requires a high level of expertise to administer.<sup>11</sup> Thus, there is an unmet need to

develop new regenerative therapies that promote the corneal wound-healing process and are capable of being readily administered outside of the surgical suite, such as in an eye drop format.

Silk fibroin is a unique protein derived from the fibers of silkworm cocoons and is considered to be a novel biomaterial for use in regenerative medicine applications.<sup>12</sup> This is primarily due to fibroin's readily controlled material properties, thermal and mechanical stability, ability to be processed into a range of material formats, and inherent biocompatible and nonimmunogenic properties when used in vivo.<sup>13</sup> Additionally, studies<sup>14,15</sup> have demonstrated the therapeutic use of fibroin-based materials to promote tissue regeneration when applied as a wound dressing. Furthermore, both in vitro and in vivo studies have been conducted that have established fibroin-derived materials as a potential biomaterial choice for corneal tissue engineering and ocular surface repair.<sup>16–20</sup> The efforts shown here are dedicated toward evaluating the effects that the presence of soluble silk fibroin-derived protein (SDP) has on the response of an in vitro corneal wound-healing model using a human corneal limbal-epithelial (hCLE) cell line. For the first time, effects of SDP presence on corneal epithelial cell migration, adhesion, and proliferation were successfully characterized in vitro.

## MATERIALS AND METHODS

### Silk Fibroin-Derived Protein Production

*Bombyx mori* silkworm cocoons were purchased from Tajima Shoji Co. (Yokohama, Japan). The silk solution was prepared from a batch of 5-g cocoons that were cut into three pieces each. The cocoons were boiled in 2 L of 0.03 M Na<sub>2</sub>CO<sub>3</sub> (Sigma-Aldrich Corp., St. Louis, MO, USA) for 45 minutes to remove the sericin protein. The extracted silk fibroin fibers were then rinsed four times in deionized water and completely dried at room temperature overnight. The dried silk fibroin fibers were then dissolved in a concentrated solution of 9.7 M LiBr (Sigma-Aldrich Corp.) for 2 hours at 60°C, and then autoclaved at 15 PSI and 121°C for 30 minutes. The solution was then dialyzed against an approximately 200X volume of water by using Snake-Skin dialysis tubing (ThermoScientific, Waltham, MA, USA) with a 3500 molecular-weight cutoff for 48 hours and six water exchanges at 1-, 4-, 8-, 12-, 12-, and 12-hour intervals. Subsequently, the dialyzed SDP solution was centrifuged twice at 10,000g for 20 minutes to remove impurities by decanting the supernatant each time. The solution was then diluted to a 50 mg/mL concentration, and stored at 4°C until use.

### Silk Fibroin-Derived Protein Molecular Weight Distribution Characterization

The MWD of SDP was evaluated by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). Specifically, 15 µg total protein content was mixed with running buffer containing SDS and dithiothreitol (BioRad, Inc., Hercules, CA, USA) to remove any potential secondary folding structures and disulfide bonds, respectively, then heated to 70°C for 5 minutes. Mixtures were loaded along with a 2.5- to 200-kDa molecular weight ladder (Life Technologies, Carlsbad, CA, USA) onto precast, 12% polyacrylamide gels containing Bis-Tris buffer salts (Life Technologies), then exposed to 120-V electric field for 90 minutes on a BioRad PowerPac Power supply. Gels were then removed and placed in Coomassie Blue stain for 12 hours to stain proteins, followed by 6 hours of washing in deionized water. Gels were then scanned on a

BioRad GS-800 Calibrated Densitometer. Sodium dodecyl sulfate–PAGE measurements were performed in triplicate to ensure consistent batch reproducibility.

### Human Corneal Epithelial Cell Culture

Human corneal limbal-epithelial cells were generously provided by Ilene Gipson (Schepens Eye Research Institute, Harvard Medical School, Boston, MA, USA).<sup>21</sup> Cells were thawed from storage in liquid nitrogen and cultured for 72 hours in keratinocyte-SFM medium (K-SFM, ThermoScientific) supplemented with 0.2 ng/mL mouse epithelial growth factor (ThermoScientific), bovine pituitary extract (ThermoScientific), 1% penicillin-streptomycin (VWR, Radnor, PA, USA), and 0.1% CaCl<sub>2</sub>·2H<sub>2</sub>O (ThermoScientific). Standard cell culture conditions (37°C, 5% CO<sub>2</sub>, >95% humidity) were used during routine passages.

### Scratch Wound Closure Assay

A scratch wound assay with hCLE cultures was used to determine if SDP could promote wound closure in vitro. Human corneal limbal-epithelial cells were seeded within 24-well plates, at  $5 \times 10^4$  cells/cm<sup>2</sup>, and allowed to incubate for a 24-hour period in K-SFM media to form a confluent monolayer. The K-SFM media was removed and the confluent cell sheet was wounded through scratching the culture well surface with a 100-µL pipette tip. The “scratch wound” creates a cell-free denuded space that the remaining culture can migrate over and mimic healing. The scratch-wounded hCLE cells were washed with 1X PBS to remove any cell fragments or detached cells before incubating in fresh K-SFM media with different SDP concentrations (0.2%, 0.4%, and 0.5% wt/vol) or PBS vehicle control, for 20 hours. Similar to previous work, PBS was selected as a negative control treatment condition for these experiments.<sup>22</sup> A globular protein control, such as bovine serum albumin, was not used as a negative control treatment, as previous work has consistently demonstrated that PBS serves as an equally effective agent in cell migration studies.<sup>23–25</sup> Cell migration was monitored by using the microscope's 24-well plate microincubator (M24 S1; PeCon, GmbH, Erbach, Germany). A Zeiss Observer Z1 microscope (Carl Zeiss, AG, Oberkochen, Germany) with ×10 objective, ×1.6 Optovar, and phase contrast filter was used to sequentially analyze the wound closure during the course of the assay. The microscope “Mark-and-Find” feature was used to memorize select positions within each well to capture multiple areas along the denuded surface. Time-lapse phase contrast imaging was used to record a frame every 15 minutes over the 20-hour incubation period with an AxioCam single-channel camera and AxioVision software (Carl Zeiss, AG).

### Analysis of Wound Closure and Cell Migration Rate

To ensure that the similar wound areas were compared, the produced wound area was traced and measured by using ImageJ software (version 1.48, <http://imagej.nih.gov/ij/>; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA) for the multiple positions within each well. Time-lapse images for each position in the wells were analyzed and the wound areas were measured to determine the percentage of wound closure at several time points throughout the course of the assay. The time points at which the wounds fully healed were recorded. The wound closure by hCLE cells in response to SDP at various concentrations was compared to that of medium containing PBS as a vehicle control. Randomly sampled single cell migratory pathways from opposing wound borders through the denuded injury area were also analyzed by using the

“Tracking” package in AxioVision software from individual time-lapse movies. The software compiled measurements for total distance, straight distance, tortuosity, and migration rate.

### Epithelial Cell Viability Assay

Human corneal limbal-epithelial cells were cultured in the presence of different SDP concentrations (0.2%, 0.4%, and 0.5% wt/vol) or PBS vehicle control, in a 96-well plate (VWR), at a cell seeding density of  $3 \times 10^3$  cells/cm<sup>2</sup>. The cultures were then subjected to the MTT colorimetric assay (ThermoScientific) per manufacturer instructions at 12 hours post treatment, which was the duration when the greatest impact of wound healing was observed in vitro. Briefly, 50  $\mu$ L MTT stock solution (5 mg/mL) was added to the cultures containing 500  $\mu$ L fresh medium and incubated at 37°C in the dark for 4 hours. After the medium was aspirated, 200  $\mu$ L dimethyl sulfoxide (Sigma-Aldrich Corp.) was added and mixed thoroughly to release the formazan. The absorbance of the resultant solution was recorded at 540 nm by using a Biomek plate reader (Beckman Coulter, Jersey City, NJ, USA). Wells containing culture media, without cells, were set up as negative controls.

### Flow Cytometric Cell Cycle Analysis

Human corneal limbal-epithelial cells were cultured in the presence of 0.4% wt/vol solution or PBS vehicle control, in a 24-well plate (VWR) at a cell seeding density of  $3 \times 10^4$  cells/cm<sup>2</sup>. The cells were then harvested at 12 hours post treatment, which was the duration when the greatest impact of wound healing was observed in vitro, and fixed in 70% ethanol for 1 hour on ice. Following fixation, samples were centrifuged and the fixative was removed before the cells were washed three times in cold 1X PBS. The fixed cells were then pelleted by centrifugation and the supernatant was decanted. Cells were then suspended in 0.5 mL FxCycle PI/RNase Staining Solution (ThermoScientific) and allowed to incubate for 20 minutes at room temperature, protected from light. Samples were then analyzed on a flow cytometer at the Weill Cornell Medical College Core Facility, using a 566-nm excitation filter and a 616-nm band pass filter to collect emission. Statistical analysis was performed by using the instrumentation FlowJo software (version 7.6.1) fitted to the Dean and Jett model as previously described.<sup>26</sup>

### Human Corneal Limbal-Epithelial Cell Migration Assay

To distinguish between the stimulatory effects of SDP on cell proliferation and cell migration, contributing to the observed acceleration in wound closure, mitotic inhibitor hydroxyurea was used to induce arrested cellular growth.<sup>27</sup> Human corneal limbal-epithelial cells were seeded within 24-well plates at  $5 \times 10^4$  cells/cm<sup>2</sup> and incubated for a 24-hour period in K-SFM media to form a confluent monolayer of cells. The cells were then incubated at 37°C with 100  $\mu$ M hydroxyurea for 4 hours to arrest cell proliferation. Medium was then aspirated and the cells were rinsed with 1X PBS. Next, a scratch assay was performed as described above to evaluate the effects of SDP (0.4% wt/vol) on accelerating wound healing through stimulation of cell migration.

### Cell Substrate Adhesion Assay

Human corneal limbal-epithelial cells were seeded in 35-mm cell culture dishes, at  $3 \times 10^5$  cells per dish. This cell seeding density was chosen to ensure proper surface coverage with minimal cell-cell contact formation. Cells were allowed to

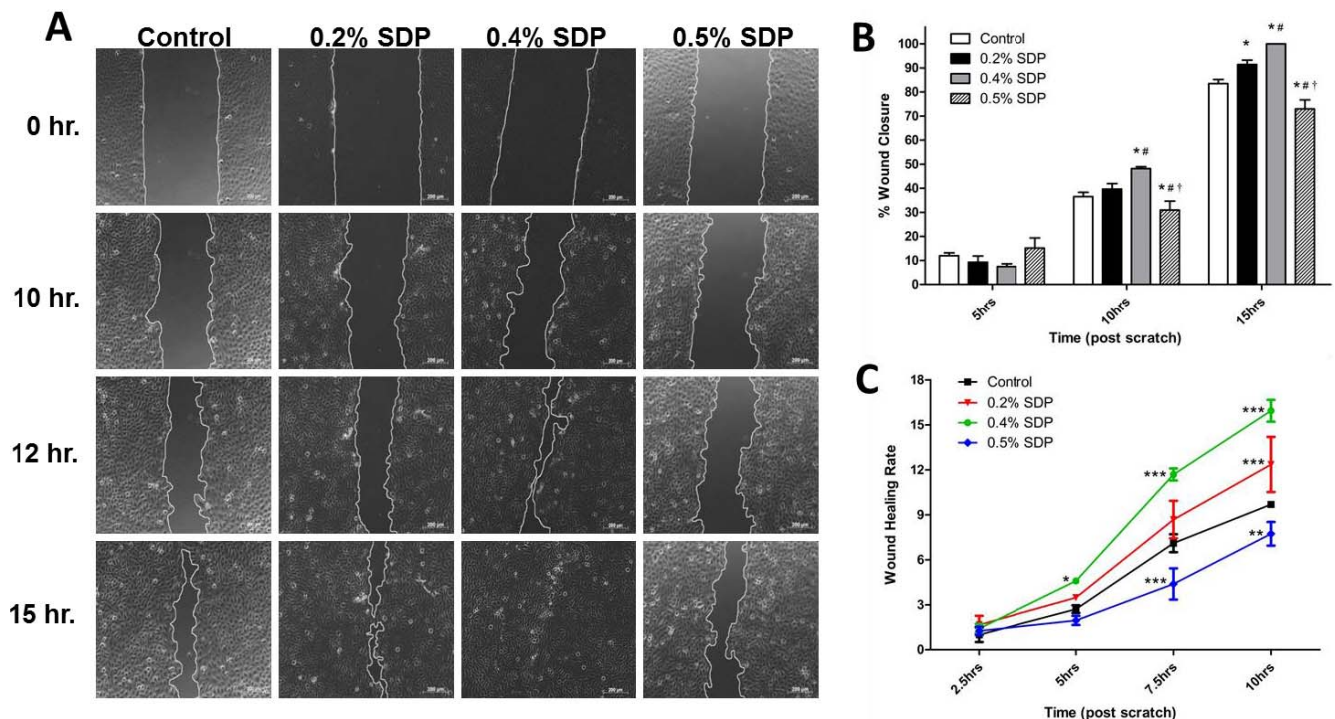
incubate overnight to ensure optimal cell attachment to the substrate. Media from the cell-seeded dishes was removed and fresh K-SFM with different SDP concentrations (0.2%, 0.4%, and 0.5% wt/vol) or PBS vehicle control was added. The cells were incubated overnight. Following incubation, cell substrate adhesion was evaluated by using a parallel plate flow chamber cell (Glycotech, Gaithersburg, MD, USA). The flow channel within the chamber had dimensions of 5-mm width, 0.1-mm height, and 48.2-mm length. Medium was aspirated from the cell-seeded dishes and the flow chamber was placed on top of the cells. Phosphate-buffered saline was warmed to 37°C to yield an apparent fluid viscosity ( $\mu$ ) of  $\sim 0.8$  cP. A syringe pump was used to deliver the PBS at a volumetric flow rate ( $Q$ ) of 52.2 mL/min to create a continuous one-dimensional laminar fluid flow within the channel.<sup>28</sup> The delivered fluid shear stress is defined by the following equation:

$$\tau w = 6\mu Q/b(b^2) \quad (1)$$

The channel width ( $b$ ) and channel height ( $h$ ) are fixed variables dictated by the dimensions of the silicone gasket attached to the flow chamber deck, which is then placed over the cultured cells. The apparent fluid viscosity ( $\mu$ ) produces a wall shear stress ( $\tau w$ ) that is a function of the volumetric flow rate ( $Q$ ) of the PBS infused by the syringe pump. Cells were subjected to a wall shear stress of 98.4 Pa for 1 minute, and areas of analysis, with a cell count of 30 to 40 cells, were imaged during the course of the assay on a Zeiss Observer Z1 microscope (Carl Zeiss, AG) with  $\times 10$  objective and phase contrast filter. Images of the cells were captured with an AxioCam single-channel camera and AxioVision software. The total number of adherent cells within the area of analysis was determined before and after the onset of flow. Cellular adhesion of cells treated with SDP was evaluated and compared to cells treated with PBS buffer (treatment vehicle) as a control.

### Human Corneal Limbal-Epithelial Immunofluorescent Staining and Imaging

Cells cultured overnight in the presence of defined concentrations of SDP (0.4%, 0.5%, and 1.0% wt/vol), or PBS vehicle control, were fixed with 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) for 15 minutes, then rehydrated in PBS containing 0.5% bovine serum albumin (Sigma-Aldrich Corp.) and 0.05% nonionic surfactant (Triton X-100; Sigma-Aldrich Corp.) for 1 hour. After fixation, 50  $\mu$ L primary antibody solution (mouse monoclonal anti-vinculin, 1:400, Sigma-Aldrich Corp.) was added for 1 hour at room temperature. Samples were subsequently incubated with secondary antibody for 1 hour by using appropriate isotype-matched nonspecific IgG as controls (ab150113, 1:500, Goat Anti-Mouse IgG H&L Alexa Fluor 488; Abcam, Cambridge, MA, USA). Samples were also stained with Alexa Fluor 568 phalloidin (1:40; ThermoScientific). After washing with PBS, samples were mounted with Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI, Vector Laboratories, Burlingame, CA, USA). Fluorescent staining was visualized by using Observer Z1 fluorescent microscope (Carl Zeiss, AG) with both  $\times 10$  and  $\times 40$  objective lenses. An AxioCam HRm digital camera and AxioVision 4.0 software were used to capture single and z-stack images (45–60 layer range) at 0.25- $\mu$ m slices, using DAPI, green fluorescent protein, and Texas Red filter channels. Deconvolution was performed on each z-stack, using 3D Huygens Deconvolution Software (Scientific Volume Imaging BV, Hilversum, The Netherlands).



**FIGURE 1.** (A) Representative images from in vitro scratch wound healing assays demonstrating that cell migration into the cell-free region (outlined) is significantly accelerated in the presence of 0.4% wt/vol SDP when compared to controls (treatment vehicle; scale bar: 200  $\mu$ m). (B) Summary bar graph illustrating percentage wound closure at indicated time points during the scratch wound assay ( $*P < 0.01$  versus control;  $\#P < 0.001$  vs. 0.2% SDP;  $\ddagger P < 0.001$  vs. 0.4% SDP;  $n = 3$ ). (C) Summary graph showing typical wound healing (migration) rates by epithelial cells in the presence of varying concentrations of SDP ( $*P < 0.05$  versus control;  $**P < 0.01$  versus control;  $***P < 0.001$  versus control,  $N = 3$  experiments,  $n = 3$  wells per treatment group).

### Cell Spreading and Cytoskeletal Reorganization

Cells cultured overnight in the presence of defined concentrations of SDP (0.4%, 0.5%, and 1.0% wt/vol) or PBS vehicle control were fixed and prepared as above described for immunofluorescence and stained under like conditions for phalloidin and DAPI. Imaging was performed as above described. The surface area of the cells, under both sparse and confluent conditions, was measured by using ImageJ software (version 1.48) to determine whether alterations to the cell spreading area had occurred in response to stimulation by SDP.

### Statistical Analysis

A 2-way ANOVA test was performed to reveal whether any of the experimental groups were affected by SDP and to identify statistically significant changes in cellular responses between treated and control groups. Post hoc Student's *t*-tests were carried out by using Prism Software (version 5.01; GraphPad Software, Inc., La Jolla, CA, USA) to determine *P* values.

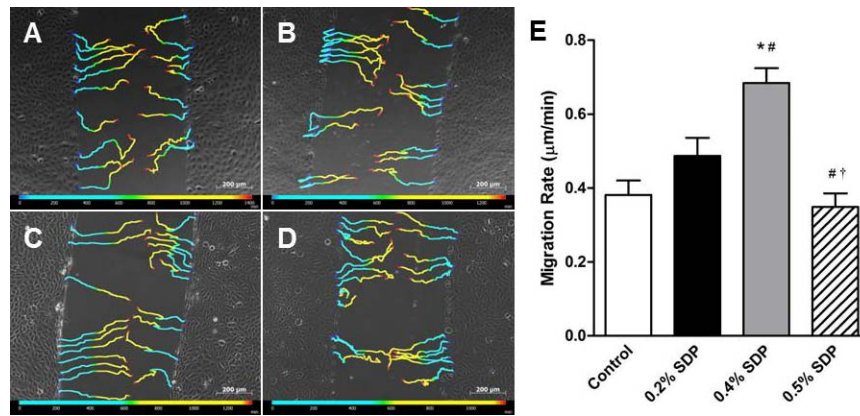
## RESULTS

### Silk Fibroin-Derived Protein Added to Culture Corresponds With Increased hCLE Cell Migration and Scratch Wound Closure

The impact of the presence of SDP on hCLE behavior in vitro was evaluated by using a scratch wound assay to assess cell migration. Human corneal limbal-epithelial cells were chosen as a reliable in vitro model of the corneal surface because of the well-characterized history of the cell line and reliability.<sup>17,29</sup>

Human corneal limbal-epithelial cells were successfully grown to confluence and then partially denuded to model a corneal abrasion. The cells were subsequently treated in the presence or absence of SDP to view and quantify the impact of the protein on the migration rate of cells into the denuded area of the culture. Epithelial growth and migration rates were successfully calculated by measuring cell coverage into the denuded space at defined time points through time-lapse imaging (Fig. 1A). An observed dose-dependent improvement in culture migration was observed in the presence of SDP, which exhibited a  $\sim 30\%$  improvement in wound closure, relative to untreated control cells, by 15 hours (Fig. 1B). The increase in epithelial cell growth and migration with SDP treatment translated into a significant reduction in scratch wound closure time relative to control cells. The cells treated with the 0.4% SDP concentration exhibited  $\sim 2$ -fold increase in healing rate by 10 hours and entirely occupied the wound area faster than control cells (Fig. 1C), indicating that the presence of SDP impacted cell migration rate in culture.

The concentration of SDP was increased to 0.5% wt/vol and the rate of wound closure was observed to be approximately 50% slower relative to cultures treated with 0.4% wt/vol SDP, and nearly 20% slower than untreated control cells at 10 hours. Cell tracking software also allowed assessment of the stimulatory effects of SDP on the motility of the border cells, which spread and crawl collectively while maintaining cell-cell contacts during invasion of the denuded "wound" area, throughout the scratch assay. Individual cell migration pathways throughout the scratch closure period were successfully tracked for all cultures (Fig. 2A). Cells treated with the 0.4% SDP concentration exhibited a 50% increase in migration rate, relative to control cells, during scratch closure (Fig. 2B). Similar to the observed decrease in scratch closure rate, cells



**FIGURE 2.** (A–D) Migratory paths of individual hCLE cells along the edge of the wound, during wound closure for (A) control, (B) 0.2%, (C) 0.4%, and (D) 0.5% SDP wt/vol. Colored tracks indicate time 0 (blue) to 15-hour time points (red) throughout the course of the assay. Scale bar: 200 µm. (E) Summary graph showing the mean singular cell migratory rate of wound border cells during scratch closure (\**P* < 0.05 versus control; #*P* < 0.05 vs. 0.2% SDP; †*P* < 0.05 vs. 0.4% SDP; *N* = 3 experiments, *n* = 20 cells evaluated per field).

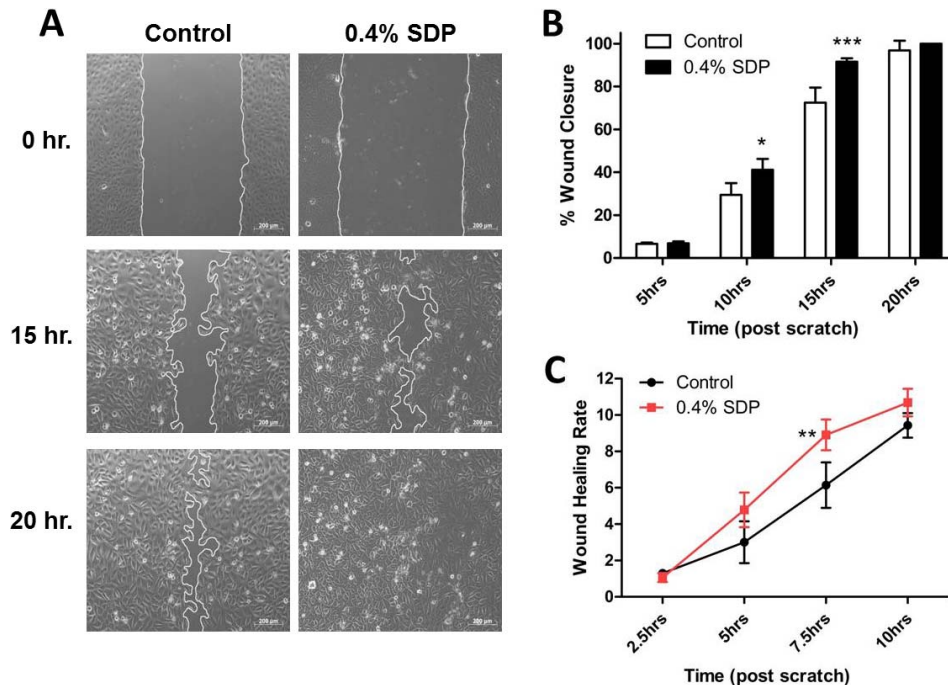
treated with 0.5% wt/vol SDP exhibited a 50% and 30% decrease in migration rate, relative to cells treated with 0.4% wt/vol and 0.2% wt/vol SDP, respectively.

To further investigate the impact SDP has on inducing cell migration during wound healing, the ability of cells to invade an acellular area was assessed after growth arrest. Human corneal limbal-epithelial cells were first cultured to form a confluent cell sheet, before being treated with the mitotic inhibitor hydroxyurea before scratch wound, either in the presence or absence of 0.4% wt/vol SDP. This concentration of SDP was chosen because previous experiments have demonstrated an optimal enhancement in cell migration and scratch wound closure above. Results indicated that wound closure was enhanced by 30% after 10 hours and 20% after 15 hours in

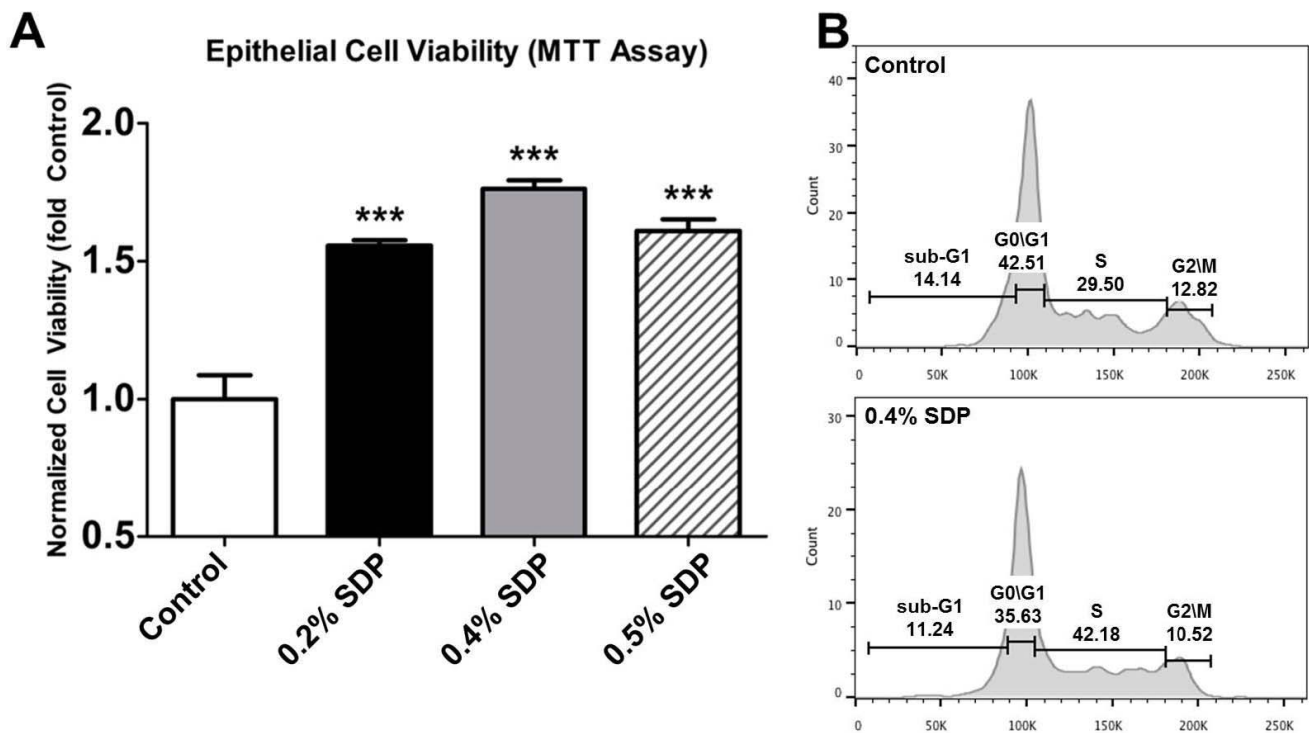
SDP-treated cultures, relative to vehicle controls (Fig. 3A). Overall wound healing rate was accelerated in cells treated with SDP, with an observed significant increase of 30% after 7.5 hours (Fig. 3C). Collectively, these results show that SDP induced increased hCLE migration in scratch wound closure assays.

### Silk Fibroin-Derived Protein Added to Culture Corresponds With Increased hCLE Cell Number and Proliferation

Studies were performed to evaluate the impact of SDP presence on hCLE cell proliferation. Human corneal limbal-



**FIGURE 3.** (A) Representative images from wound healing assay of hCLE cell cultures treated with a mitotic inhibitor, hydroxyurea, demonstrated that cell invasion into the cell-free region (outlined) is accelerated in the presence of 0.4% wt/vol SDP compared to control (treatment vehicle; scale bar: 200 µm). (B) Summary bar graph illustrating percentage wound closure at indicated time points during the scratch wound assay (\**P* < 0.05 versus control; \*\*\**P* < 0.001 versus control). (C) Summary graph showing typical wound healing rates by hCLEs in the presence of 0.4% wt/vol SDP or treatment vehicle control (\*\**P* < 0.01 versus control, *n* = 3).



**FIGURE 4.** (A) Summary graph of MTT viability assay performed on cells cultured in the presence of increasing concentrations of SDP or treatment vehicle (control) over a 12-hour period. All concentrations of SDP significantly increased proliferation, relative to control cells. (\*\*\*)  $P < 0.001$  versus control,  $n = 3$ ). (B) Cell cycle distribution histograms of cells cultured in the presence of 0.4% wt/vol SDP, compared to control (treatment vehicle), and stained with PI showing DNA content distribution. The G1 and G2 phase histogram peaks are separated by the S phase distribution. Treatment with SDP resulted in an increased S phase distribution of the cell cycle, relative to control cells.

epithelial cells were successfully cultured in like numbers in the absence or presence of increasing concentrations of SDP for 12 hours. The cultures were then subjected to an MTT colorimetric metabolic assay to measure the change in cell number (Fig. 4). Relative to nontreated cells (control), SDP enhanced cell growth by over 50% for all concentrations, in which increased metabolic activity corresponds to increased cell proliferation. Flow cytometric analysis was subsequently performed to evaluate the distribution of cell populations in various phases of the cell cycle to further elucidate the effects of SDP on cell proliferation. hCLE cells were first cultured in like numbers in the presence or absence of 0.4% wt/vol SDP solution for 12 hours. This concentration of SDP was chosen because it demonstrated the greatest increase in epithelial cell growth, measured by MTT colorimetric assay. Cells were harvested and fixed before staining with propidium iodide (PI) for DNA content cell cycle analysis by using flow cytometry (Fig. 4). Relative to nontreated cells (control), treatment with 0.4% wt/vol SDP increased the distribution of cells in the synthesis (S) phase of the cell cycle by nearly 50%. Increased cells in S phase correspond to increased cell proliferation.

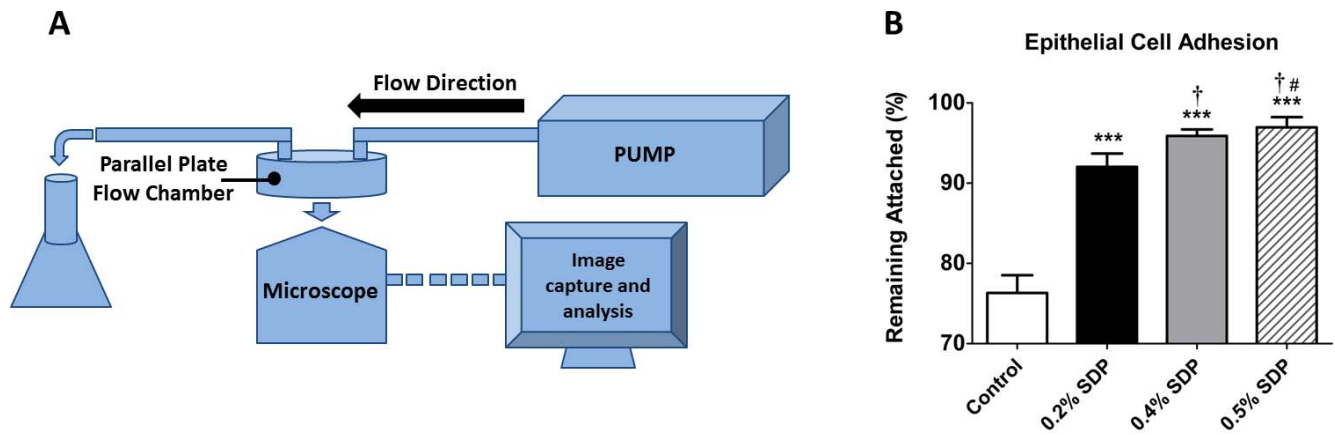
#### Silk Fibroin-Derived Protein Presence Corresponds to hCLE Cell Spreading, Cell-Substrate Adhesion, and Increased Focal Adhesion Formation

The impact that the presence of SDP in culture had on epithelial cell adhesion strength to the underlying substrate was successfully assessed. The laminar fluid flow chamber was able to evaluate the resistance of SDP-treated cells to detach from their substrate when challenged by fluid shear stress. Human corneal limbal-epithelial cells were cultured on 2D

surfaces and then placed in the laminar flow chamber for 1 minute. The integrated flow circuit continuously imaged the various cultures, and the images were used to quantify cell detachment in response to fluid shear (Fig. 5A). Untreated control cultures exhibited an approximate 75% retention of cells under these conditions (Fig. 5B). However, treatment with 0.2% SDP significantly increased cell retention to over 90%, indicating enhanced cell-matrix adhesion in the presence of SDP. Furthermore, improvements to cell attachment were dose-dependently related to protein concentration. Cells treated with 0.5% SDP exhibited over 95% retention upon being subjected to high shear.

Focal adhesion (FA) formation by hCLE cultures was successfully assessed by immunofluorescence-based microscopy of vinculin protein staining in the absence or presence of SDP. Vinculin is a key component of this complex and is a reliable indicator of FA presence and location.<sup>30</sup> In untreated control cell cultures, hCLEs displayed a dispersed arrangement of vinculin throughout the cell periphery (Fig. 6A), which became more numerous at the plasma membrane surface with 0.4% and 0.5% wt/vol SDP (Figs. 6B, 6C) and was found to enhance cell adhesion the most during the previous experiments. Additionally, increased vinculin aggregates formed with increasing SDP concentration to 1.0% wt/vol (Fig. 6D), indicating increased FA formation and clustering had taken place.

Human corneal limbal-epithelial spreading was successfully assessed in the presence or absence of defined SDP concentrations for both single cells and confluent monolayers to mimic an environment adjacent to a wound and also on an uninjured corneal surface. Cells cultured at low densities possessed a larger average surface area as compared to cells in a compact monolayer (Fig. 6E). Incubation of SDP with both low and high cell densities evoked a robust increase in cell



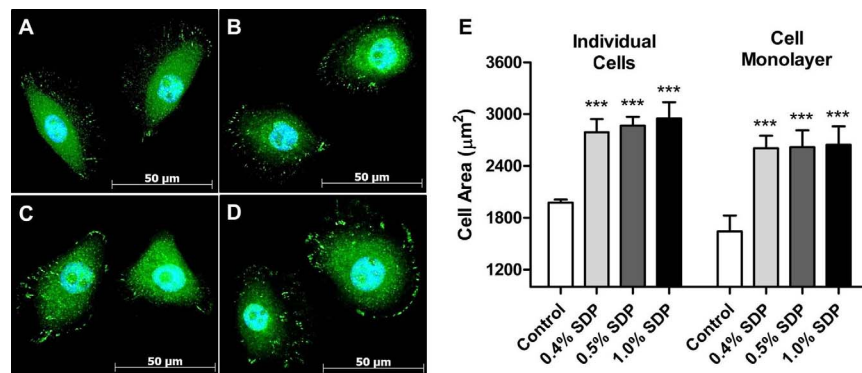
**FIGURE 5.** Silk fibroin-derived protein improves cell adhesion during fluid shear. **(A)** Schematic of integrated flow circuit, image capture, and analysis system. Fluid is infused by the syringe pump into the parallel plate flow chamber to yield a uniform level of shear force upon the substratum within the flow channel. Microscopic images of cells are sequentially captured throughout the course of the assay and subsequently analyzed to quantify cell detachment. **(B)** Summary graph demonstrating a significant increase in hCLE cell attachment to standard plastic tissue culture dishes during high fluid shear ( $\sim 98.4$  Pa), following preincubation with aqueous SDP dose dependently ( $***P < 0.001$  versus control;  $\dagger P < 0.01$  vs. 0.1% SDP;  $\#P < 0.05$  vs. 0.2% SDP,  $N = 3$  experiments,  $n = 100$  cells evaluated per treatment group).

surface area, indicative of increased cell attachment. Observed cell response was not dose dependent, as increasing the concentration of SDP did not result in any significant increases in cell spreading.

## DISCUSSION

There is a growing need for the development of novel therapies to treat ocular surface injuries and improve clinical outcomes for suffering patients. Silk fibroin protein has been shown to have stimulating effects on wound healing,<sup>31,32</sup> and in this study, soluble SDP added to cell culture media induced a significant increase in migration of hCLE cells, contributing to an accelerated rate of epithelial sheet wound closure in an in vitro scratch wound model of the corneal epithelium. The physical closure process of an ocular surface wound is driven primarily by increased cell proliferation and leader cell migration at the wound edges.<sup>33</sup> The results above support that SDP may directly enhance wound closure by stimulating both epithelial proliferation and positively impacting migration rate.<sup>31</sup>

In addition to cell proliferation and migration, proper cell attachment to an underlying basement membrane matrix is crucial for corneal epithelial wound healing.<sup>4</sup> Healthy spreading and adhesion are required for clear vision and maintenance of an external barrier for the posterior corneal tissue.<sup>34</sup> Integrin proteins are the extracellular tethering components of cellular FA complexes, which bind to protein receptors existing within the local microenvironment, such as the arginine-glycine-aspartic amino acid (RGD) sequence found in basement membrane protein fibronectin.<sup>35</sup> It is known that *Bombyx mori* fibroin protein does not contain previously identified integrin-binding receptors (e.g., RGD, YIGSR); however, films formed from SDP have been shown to promote corneal epithelial cell attachment and growth.<sup>36,37</sup> Human corneal limbal-epithelial cells cultured in the presence of SDP exhibited an increase in cell spreading and FA formation with increasing SDP concentration. In addition, cell-surface attachment force was significantly enhanced with increasing SDP concentration. Collectively, these results indicate that SDP is having an impact on increasing FA formation, and therefore increased cell attachment strength. Furthermore, the data



**FIGURE 6.** (A–D) Silk fibroin-derived protein enhances cell adhesion by promoting FA formation and clustering. Representative images of hCLE cells cultured in (A) control conditions and in the presence of (B) 0.4%, (C) 0.5%, or (D) 1% wt/vol SDP incubation for 16 hours, showing increased vinculin (green) staining along the cell membrane, indicating points of cell FA attachment (nucleus = blue). Cells also exhibited larger vinculin clusters with increased SDP concentration, relative to control cells. (E) Human corneal limbal-epithelial spreading is enhanced in the presence of SDP. Summary graphs represent mean surface area of hCLE cells cultured sparsely (individual) or to confluence (monolayer) as would occur on the intact ocular surface, in the presence of different concentrations of SDP, or treatment vehicle ( $***P < 0.001$  versus respective  $N = 3$  experiments,  $n = 100$  cells evaluated per field).

above suggest that high SDP concentrations (i.e., >0.4%) increase cell attachment strength to the extent that migration is impaired.

In summary, findings in this study demonstrated that the presence of SDP in vitro influences hCLE behavior by way of increasing cell migration, proliferation, and adhesion. Further research is required to identify the mechanisms and associated signaling pathways through which SDP may impact cell proliferation and migration, and to elucidate the mechanistic underpinnings behind the impact SDP presence is having on FA complex formation and clustering regulation. In addition, future effort will be devoted to identifying the biological mechanisms by which SDP may actively promote the corneal epithelium's wound-healing process, and to further characterize the biochemistry contained in the SDP MWD to resolve structure-function relationships. In total, these observations support further consideration of SDP as an ingredient in eye drop formulations for the treatment of ocular surface injury, especially as it pertains to disease states including persistent epithelial defects or ocular surface injuries where enhanced epithelial cell migration and adhesion are required for improving clinical outcomes.

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