Characterization of Partially Lifted Cell Sheets

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There is a need to characterize biomechanical cell–cell interactions, but due to a lack of suitable experimental methods, relevant *in vitro* experimental data are often masked by cell–substrate interactions. This study describes a novel method to generate partially lifted substrate-free cell sheets that engage primarily in cell–cell interactions, yet are amenable to biological and chemical perturbations and, importantly, mechanical conditioning and characterization. A polydimethylsiloxane (PDMS) mold is used to isolate a patch of cells, and the patch is then enzymatically lifted. The cells outside the mold remain attached, creating a partially lifted cell sheet. This simple yet powerful tool enables the simultaneous examination of lifted and adherent cells. This tool was then deployed to test the hypothesis that the lifted cells would exhibit substantial reinforcement of key cytoskeletal and junctional components at cell–cell contacts, and that such reinforcement would be enhanced by mechanical conditioning. Results demonstrate that the mechanical strength and cohesion of the substrate-free cell sheets strongly depend on the integrity of the actomyosin cytoskeleton and the cell–cell junctional protein plakoglobin. Both actin and plakoglobin are significantly reinforced at junctions with mechanical conditioning. However, total cellular actin is significantly diminished on dissociation from a substrate and does not recover with mechanical conditioning. These results represent a first systematic examination of mechanical conditioning on cells with primarily intercellular interactions.

Introduction

THE RECENT DEVELOPMENT of cell-sheet tissue engineering has generated a need for a systematic characterization of cell–cell interactions in cell sheets to better mimic and condition them for *in vivo* applications. Rather than using conventional three-dimensional scaffolds for tissue reconstruction, an approach using thermo-responsive polymeric surfaces that facilitate the noninvasive harvest of cultured cells as intact tissue sheets was developed.¹ Such cell sheets have been generated for a wide variety of laminar tissues, such as skin, heart, corneal, and renal components.^{2–5} In addition, cell sheet tissue engineering bears a striking resemblance to the embryonic cell sheet building machinery. In early development, embryonic morphogenesis results largely from deformation of analogs of cell sheets, via internally generated forces.⁶ As a naturally existing cell sheet, the blastoderm consists of a layer of cells that are enclosed in a fluid-filled blastocoel cavity, lacking extracellular matrix (ECM) support.⁷ The rearrangement and deformation of the cell layer in blastoderms and later in blastopores involves a series of precisely orchestrated morphogenetic episodes.^{8,9} The parallels between tissue engineering and tissue morphogenesis suggest that force homeostasis across cell–cell junctions not only govern blastoderm and blastopore formation, but also may play crucial roles in regulating

mechanical strength of the cell sheet constructs for tissue engineering purposes.

Currently, cell sheets are fragile and are typically handled by external supports.¹⁰ Direct experimental methods for understanding and improving the sheets' biomechanical properties, such as cell–cell adhesion, mechanotransduction, and other baseline cellular properties, are essential for further development of these sheet constructs. However, comprehensive *in vitro* experimental data are still lacking due to lack of suitable experimental methods. First, research in cell sheet engineering primarily focuses on biological or chemical cues; comparatively little is known about mechanical cues. In particular, how mechanical cues may regulate, or be regulated by, the cytoskeleton remains incompletely resolved. Since components such as actin are responsible for certain mechanoresponses as well as for cell processes such as migration, contraction, and adhesion, it is imperative that their role be examined in more detail.^{11–17} Second, most studies are done in adherent cells that may primarily maintain cell–substrate interactions and, as a result, they likely introduce mixed responses into the readouts. Thus, the roles of key junctional proteins in desmosomes, adherens junctions, and so on are not well characterized. However, recent studies have demonstrated that such junctional proteins regulate a variety of processes such as viability and migration.^{18–20} Third, most cell sheets are

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generated for immediate use and not conditioning—without supporting scaffolds, these sheets are too fragile to endure *in vitro* handling or significant manipulation. A recent study on characterizing the mechanics of cultured cell monolayers has begun shedding light on this topic.²¹ By culturing cells on a sacrificial collagen scaffold between test rods, and subsequently dissolving away the scaffold, the investigators provided a novel method to measure monolayer elasticity and ultimate strength. Despite this excellent work, our knowledge of underlying mechanisms regarding how cell–cell junctions and cytoskeleton regulate cell sheet mechanical properties, and, more importantly, the capability to mechanically condition the cell sheet for tissue engineering purposes remain poor.

In this study, we developed a novel method to generate partially lifted cell sheets that can be manipulated in a way similar to adherent cells. This simple yet powerful tool enables us to investigate the effects of mechanical conditioning on cell sheet properties and permits a direct comparison of physiological parameters between lifted cells and their adherent controls, side by side. We hypothesized that lifted cells would exhibit changes to the distribution of cytoskeletal and junctional proteins, with reinforcement occurring at cell–cell contacts. We further hypothesized that mechanical conditioning would enhance such reinforcement, as well as lead to greater cohesion in the lifted cells. To test these hypotheses, we characterized cellular actin and junctional reinforcement, and evaluated cell sheet strength and cohesion, to assess changes in cell properties when cell–cell interactions dominate. We found that our hypotheses are generally supported, but that there is a limit to reinforcement via mechanical conditioning.

Materials and Methods

Cell culture

Immortalized human keratinocytes (N/TERT-1) were maintained as described elsewhere.^{18,22} Briefly, cells were plated on 0.005" thick silicone sheeting (SMI, Saginaw, MI) in a 100-mm diameter custom-made stretch device chamber, expanded and propagated in keratinocyte serum-free media (abbreviated ker-sfm, media, and supplements from Invitrogen, Carlsbad, CA, unless otherwise stated), and supplemented with recombinant epidermal growth factor (rEGF) (0.2 ng/mL) and BPE (25 µg/mL), CaCl₂ (0.4 mM; Sigma, St. Louis, MO), and penicillin/streptomycin. To grow cells to high confluency, cells were switched to a medium consisting of a 1:1 mixture of ker-sfm and a medium DF-K, with the latter consisting of a 1:1 mixture of Dulbecco's-modified Eagle's medium (DMEM) and Ham's F-12, supplemented with rEGF (0.2 ng/mL) and BPE (25 µg/mL), L-glutamine (1.5 mM), and penicillin/streptomycin.

Antibodies and reagents

Unless otherwise noted, reagents were purchased from Invitrogen/Life Technologies (Carlsbad, CA). Primary mouse monoclonal antibodies anti-plakoglobin (γ -Catenin) and anti-GAPDH (Novus Biologicals, Littleton, CO) were used for immunoblotting. Immunofluorescence staining was performed using the anti-plakoglobin antibody as the primary antibody and Alexa Fluor 594 goat anti-mouse IgG as

the secondary antibody. Alexa Fluor 488-conjugated phalloidin at a concentration of 0.5 µg/mL was used for actin staining. Hoechst was used at a concentration of 0.5 µg/mL for nuclear staining. Cytochalasin D (CytoD; Sigma) was used to disrupt actin at 3 µM for 1 h right after sheet lifting, and Y-27632 was used to inhibit Rho-associated kinase (ROCK) at 10 µM for 1 h right after sheet lifting.

Dispase-based partial-lift method

Cells grown to full confluence were treated with dispase at a concentration of 2.4 units/mL in Hanks-Buffered Saline Solution inside the custom-made polydimethylsiloxane (PDMS) mold (with inner diameter 10 mm), and incubated at 37°C for 30 min until the monolayer visibly lifted from the chamber as an intact sheet. Cells outside the PDMS mold remained adhered to the substrate. Thus, this plating is considered a partially lifted culture, where the cells within the PDMS mold form a lifted cell sheet that was dissociated from the substrate but were attached at the periphery to the cells which remained plated.

Fluorescence microscopy

Cells were fixed in 4% paraformaldehyde (Sigma) and then permeabilized with 0.1% triton-X-100 (Sigma). Cells were then incubated in the primary antibody, phalloidin, or Hoechst for an hour, followed by phosphate-buffered saline washes and then, for samples tagged with a primary antibody, a secondary antibody incubation for another hour and washed again. Cell viability was determined using a Live/Dead cell viability assay (Invitrogen) according to the manufacturer's instructions.

Microscopy was performed at room temperature using an Olympus FV10 Confocal microscope, an Olympus 40× NA 1.3 objective, and Olympus FV10-ABW Software. Images were processed using ImageJ (version 1.43u; National Institutes of Health, Bethesda, MD) and scaled down in Photoshop (Adobe).^{23–25} Relative actin or plakoglobin intensities were quantified by ImageJ, with values normalized to untreated control (i.e., unstretched, unlifted cells) signals. The percentage of actin or plakoglobin signal at the junctions was calculated as signal intensity at the junctions divided by the total signal intensity of the whole cells using previously described methods.^{26,27} Images were acquired under identical conditions, and analyses were performed in raw images. Images for figures were colored and adjusted for brightness and contrast.

Equiaxial cyclic stretch, stretch verification test, tensile test, and shear test

Partially lifted cell sheets were primarily subjected to equiaxial cyclic stretch for 4 h at 5% strain and 1 Hz before staining, using a custom equiaxial stretching device (with uniform strain fields, as previously described²⁸) maintained in a cell culture incubator.

Tensile test assays were performed using an Instron ElectroPuls E1000 All-Electric Test Instrument (Instron, Norwood, MA). Strain was calculated as the ratio of the change in length to the original length ($\epsilon = \Delta L/L_0$), and the maximum sheet strain before failure was measured. A previously established shear test protocol was performed to

test cell sheet cohesion and, thus, cell–cell adhesive strength.^{18,26,29,30}

A second custom equiaxial stretch apparatus, referred to as the microscope stage-top stretch apparatus, was used for simultaneous stretching and imaging. This device consisted of a moving plate that is moved vertically by an actuator against a ring-shaped piston which generates uniform strain on the membrane. A microscope objective can fit within the piston to image the membrane, and this device was used to measure strains of the cell sheets and to verify that cells in the lifted regions were subject to the same strain as cells which remain attached. Nuclei were labeled using a Hoechst stain and imaged during stretch. Nuclei centroids were tracked in multiple fields of view, and their coordinate positions were determined using an automated particle-tracking Matlab program as previously described.^{31,32} The lengths between multiple randomly picked nuclei were measured. Strain was calculated as for the tensile test.

siRNA knockdown of plakoglobin

Silencer[®] Pre-designed siRNA for plakoglobin (5′-3′: GGGCAUCAUGGAGGAGGAUtt), and a negative control siRNA (Invitrogen) were used. Cells were transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions. Cells were harvested and analyzed for 4 days after transfection.

Immunoblotting

Total protein concentration was determined by Bradford assay (Sigma). Soluble fractions containing equal amounts of total protein were separated using sodium dodecyl sulfate–polyacrylamide gel electrophoresis and transferred onto PVDF membranes (Millipore, Billerica, MA). Immunoblotting was performed using mouse anti-human antibodies at the following dilutions: anti-Plakoglobin 1:2000, anti-GAPDH 1:1000, and horseradish peroxidase-conjugated goat anti-mouse secondary antibody 1:1000. Blots were developed with ECL reagents (Perkin Elmer, Waltham, MA) and imaged using a FUJI imaging unit (Fujifilm, Stamford, CT). Relative plakoglobin intensity in immunoblotting images was quantified by ImageJ, with values normalized to GAPDH of each matching blot lane and the signal of the untreated control.

Statistical analysis

Data are expressed as mean $\pm$ SD and compared by ANOVA and Tukey's post-test as appropriate. A value of $p < 0.05$ was considered significant, with each group having sample sizes of $n \geq 3$.

Results

Generation of partially lifted cell sheets

To examine and manipulate cell sheets that have minimal or no interaction with substrates, we developed a method to generate partially lifted cell sheets. Confluent human keratinocyte monolayers plated on a flexible silicone membrane were lifted by dispase inside a PDMS mold that was gently placed on top part of the culture area. Cells outside, and under, the PDMS mold remained attached to the membrane

(Fig. 1A). The lifted cell region inside the mold, thus, consisted of a cell sheet that engaged mostly cell–cell interactions, yet was available for immunofluorescence staining and microscopy, and importantly, could be mechanically conditioned due to their attachment to cells which remain attached to the substrate. To confirm the effectiveness of the PDMS mold in making a nonleaking well and determine whether cells in the lifted region were no longer significantly interacting with the substrate, nuclei staining was first performed across the whole monolayer, followed by partial lift with dispase, and, finally, actin staining with phalloidin inside the mold only. Only cells inside the PDMS well, that is, lifted cells, were actin stained, whereas the cells under and outside the well remained un-stained. Nuclei were stained throughout the membrane. This result confirmed that the PDMS well has the ability to contain the dispase and phalloidin solutions within the well and that the attached cells under the PDMS mold were not removed. Cells in the lifted region displayed a morphology that was consistent with fully detached cell sheets, exhibiting actin primarily at cell–cell junctions (Fig. 1B).¹⁴ The attached cells (top half) of the nucleus stain image are slightly out of plane, further confirming that the attached cells under PDMS were not removed, and suggesting that the cells inside the well have lifted (bottom half). Next, about 75% of the perimeter of the lifted region was purposely torn, resulting in significant contraction of the lifted sheet. The lifted region was also clearly not attached to the substrate, as it was easily shifted around, unlike the regions that remained attached.

To verify that cells in the lifted regions were subject to the same strain as the controls, we used a custom-made microscope stage-top stretch apparatus which is capable of simultaneous stretching and imaging. The partially lifted sheets were imaged, stretched to 9%, and imaged again. Nuclear centroids were tracked, and their coordinate positions were determined using a Matlab program. The changes in lengths between nuclei, normalized to the baseline lengths (i.e., the strains), were measured in multiple fields of view. The representative images showed measurements of changes in length between three randomly picked nuclei. No significant differences between the lifted sheets ($8.5\% \pm 1.1\%$) and controls ($8.8\% \pm 1.4\%$) were observed ($p > 0.05$, $n = 3$, Fig. 1C). Other strains (from 0% to 12%) displayed similar results (higher strain data were presented, as differences are likely to be clearer at higher strains).

To ensure that neither lifting nor the application of cyclic stretch would lead to cell death, viability assays were applied to cells under four conditions: lifted or attached, and after cyclic stretch or unstretched. Cyclic mechanical stretch was performed using an equiaxial stretch device for 4 h at 5% strain and 1 Hz. Cells were effectively 100% viable, with no significant differences among the four tested conditions (Fig. 1D). A viability test was also done on cells located under the PDMS mold with similar results, indicating that the application of the mold did not significantly damage the cells underneath it. Further, cell viability was independent of location on the membrane at each of the other strains tested in this study (Supplementary Fig. S1; Supplementary Data are available online at www.liebertpub.com/tea).

FIG. 1. Generation of partially lifted cell sheets. **(A)** Schematic illustration (side-view) of the partially lifted cell sheet (1) before lifting, (2) after lifting, and (3) subject to cyclic stretch. Confluent keratinocyte monolayers were completely lifted by dispase inside the polydimethylsiloxane (PDMS) mold, leaving the lifted region freely suspended; cells outside the mold remained adhered to the substrate. Changes in actin (green) and plakoglobin (red) indicate anticipated changes in relative levels of the respective proteins in response to stretch. **(B)** Schematic illustration (middle, top view), and enlarged fluorescent actin stain and nuclei stain images (left) on the border of the partially lifted cell sheet. Nuclei stain was performed across the whole monolayer, whereas partial lift and actin staining were performed inside the PDMS well. Only lifted cells inside the well were actin stained; the cells under and outside the well exhibited only nuclei staining. This result confirmed that the PDMS mold had the ability to contain the dispase and/or phalloidin within the well, and the attached cells under the PDMS mold were not torn off. The right image shows a partially torn cell sheet, for assessing detachment, with apparent contraction (with three edges detached to assess contraction and attachment to the substrate). These images also provide evidence to confirm the effectiveness of dispase lifting. **(C)** Nuclei tracking was used to verify that lifted sheets were subject to the same strain as controls. The representative images show changes in length between pairs of three randomly picked nuclei centroids. No significant difference was observed between the attached and lifted sheets. **(D)** Viability testing show that cells are effectively 100% viable, with no significant differences among the six tested conditions (unstretched control and 5% strain). Please refer to Supplementary Figure S1 for additional viability tests at 3%, 10%, and 20% strains. Color images available online at www.liebertpub.com/tea

Stretch induces reinforcement of lifted cell sheet cohesion

We next assessed whether mechanical conditioning can significantly alter biomechanical properties such as cell–cell adhesion strength. We chose to use stretch for mechanical conditioning, as stretch is a well-known potent stimulus for various functions, such as growth, remodeling, and gene expression. Interestingly, the effects of stretch conditioning on cell–cell adhesion of lifted sheets have not been directly measured. Two tests were used to assess cell–cell adhesion strength: (i) a tensile test that consisted of slowly stretching the cell sheet uniaxially until the sheet (or membrane) breaks, and (ii) a shear test which consisted of removing and shearing the lifted region and measuring the shearing time until the sheet fragments. The former represents a measure of the maximum tensile strain of the cell sheet, and the latter represents a measure of overall cell sheet cohesion (the overall strength). Both tests were carried out on lifted sheets stretched at 3%, 5%, 10%, and 20% strain for either 4 or 24 h, and compared with matched unstretched lifted sheets (for clarity, while the tensile test involves stretching, further references using the term “stretch” refer to mechanical conditioning, while the tensile test will be referred to by

name and the corresponding tensile test results will be referred to as “extension”). Mechanical conditioning at 3% and 5% strain resulted in a 68% and 60% increase in maximum sheet extension before failure in the tensile test at 4 h (Fig. 2A), and a 77% and 87% increase in sheet shearing time (Fig. 2C) at 4 h, respectively. Similarly, mechanical conditioning at 3% and 5% strain resulted in a 73% and 82% increase in maximum sheet extension before failure in the tensile test at 24 h (Fig. 2B), and a 112% and 93% increase in sheet shearing time (Fig. 2D) at 24 h, respectively. However, increasing strain to 10% and 20% resulted in no significant increase in cell–cell adhesion strength, and, in fact at 24 h, this strength actually decreased compared with unstretched controls for 20% strain. These results quantitatively demonstrate that mechanical stretch regulates sheet mechanical properties when cell–cell interactions dominate.

Actin distribution is significantly altered in lifted cell sheets

Since cell–cell adhesion is increased with mechanical conditioning, we next examined actin expression, working from previous data showing that the actin cytoskeleton is an essential contributor to cell sheet cohesion. To determine the

FIG. 2. Stretch induces reinforcement of substrate-free sheet strength and cohesion. Tensile tests show that stretch conditioning at 3% and 5% strains resulted in a significant increase in maximum sheet extension before failure at (A) 4 h and (B) 24 h, normalized to unstretched controls, respectively. However, increasing strain to 10% and 20% resulted in no significant increase in maximum sheet extension, and, in fact, at 24 h and 20% strain, it significantly decreased compared with unstretched controls. The shear tests show a significant increase in sheet cohesion strength (sheet broken time) after stretch at (C) 4 h and (D) 24 h, normalized to unstretched controls, respectively. Similarly, increasing strain to 10% and 20% resulted in no significant increase in cell–cell cohesion strength, but decreased compared with unstretched controls for 20% strain at 24 h. * $p < 0.05$, ** $p < 0.01$. Color images available online at www.liebertpub.com/tea

FIG. 3. Actin localization and expression is altered in mechanically conditioned lifted cells. (A) Fluorescence images of actin in unstretched control (top left), unstretched lifted (top right), stretched control (bottom left), and stretched lifted (bottom right) show that the spatial arrangement of the actin cytoskeleton is significantly altered when cells are lifted or mechanically conditioned. Green: actin, Blue: nucleus. Scale bar: 10 μ m. (B) Quantification of the percentage of cortical actin, showing highest levels of cortical actin in cells that are both lifted and stretched. (C, D) Western blot analysis shows significant up-regulation of total cellular actin in response to stretch in adherent, but not lifted, cells. Significant decrease in total cellular actin occurred after losing cell–substrate adhesion. The y-axis represents relative actin expression level, with values normalized to the unstretched control. GAPDH was used as a loading control. * $p < 0.05$, ** $p < 0.01$. Color images available online at www.liebertpub.com/tea

changes to actin expression and distribution in mechanically conditioned cell sheets, we stained cells under four different experimental conditions—attached or lifted; and with or without cyclic mechanical stretch conditioning. Unstretched attached cells generated stress fibers throughout the cell. After losing cell–substrate contact, the lifted cells lost cytoplasmic stress fibers and exhibited primarily cortical localization of actin at cell–cell contact regions (Fig. 3A, top row).

In response to cyclic stretch, adhering cells exhibited stronger overall staining for actin, while cells in the lifted regions exhibited stronger cortical staining of actin (Fig. 3A, bottom row). Quantification of the fluorescence signal at cell–cell junctions showed that the percentage of cortical actin was significantly increased from 39% to 60% after being lifted, or to 52% after being stretched while attached (Fig. 3B). Stretched lifted cells exhibited the highest percentage of cortical actin at 74%. Stretching and/or lifting lead to significant increases in actin at, or near, cell–cell junctions, which may contribute to increased cell–cell adhesive strength.

We next measured the level of total cellular actin, under the same conditions of lifting and stretching. Western blot

analysis revealed that the loss of cell–substrate contact resulted in a 79% decrease in the total expression of actin (Fig. 3C, D). Cyclic stretch caused a 55% up-regulation of actin in cells that remained attached; however, actin levels were not significantly altered by stretch in the lifted cells. Thus, for cells in the substrate-free region, both the spatial arrangement and expression level of actin cytoskeleton are significantly altered when cell–cell interactions dominate. These results suggest that the majority of actin is used for establishing and maintaining components for adhesion to the substrate.

Actin or ROCK disruption mechanically weakens lifted cell sheets

Actin-myosin coupling participates in force generation among neighboring cells. To determine whether intact actin or myosin regulates the cohesion of lifted cells, CytoD or Y-27632 (a selective inhibitor of ROCK) was used to disrupt those processes, respectively. Each type of disruption substantially altered actin distribution in lifted cells, leading to diffuse distribution of actin throughout the cell (Fig. 4A). Tensile and shear testing showed that disruption of either

FIG. 4. Actin or actin-myosin disruption weakens lifted cell sheet cohesion. **(A)** Fluorescence images of actin-stained lifted sheets, in unstretched control (top left), unstretched cytochalasin D (CytoD) treated (top middle), and unstretched Y27632 treated (top right), stretched control (bottom left), stretched CytoD treated (bottom middle), and stretched Y27632 treated (bottom right). Scale bar: 10 μ m. **(B)** Tensile tests show that treatment with CytoD or Y27632 led to significant reductions in maximum sheet strain before failure. **(C)** Shear tests shows significant diminishment in sheet cohesion with treatment of CytoD or Y27632. * $p < 0.05$, ** $p < 0.01$ compared with the matched untreated control. Color images available online at www.liebertpub.com/tea

actin or myosin interactions weakened lifted sheet maximum strain (Fig. 4B, a 71% and a 50% reduction, respectively) as well as sheet cohesion (Fig. 4C, a 60% and a 56% reduction, respectively). The increased fragility of the lifted cells was apparent when mechanical conditioning led to significant tears in the lifted region before any measure-

ments could be made. These results confirm the importance of actin and myosin interactions in maintaining cell sheet strength and cohesion, and provide the first quantitative data of ultimate mechanical strain for cell-cell adhesion under these treatment conditions.

Plakoglobin expression is significantly altered in lifted cell sheets

One of the major functions of certain cell-cell junctions is to glue cells together and connect the cytoskeletons of adjacent cells.^{18,33–36} Plakoglobin, a key molecular constituent that resides in both adherens junctions and desmosomes, is essential for maintaining cellular viability in substrate-free conditions.¹⁸ Further, plakoglobin exhibits stretch-induced up-regulation under conventionally plated conditions.^{26,37} Plakoglobin was, thus, examined using the same four conditions as actin to assess its role in modulating cell properties when cell-cell interactions dominate. After losing cell-substrate contact, the lifted cells exhibited primarily junctional localization of plakoglobin, which appears to colocalize with actin (Fig. 5A, B). After cyclic stretch for 4 h at 5% strain and 1 Hz, adhering cells significantly increased plakoglobin throughout the cell. Stretched lifted cells exhibited enhanced junctional immunoreactive signal, again appearing to colocalize with actin (Fig. 5A, B). The percentage of plakoglobin's immunoreactive signal at the junctions was significantly increased from 62% in unstretched control cells to 89% after being lifted, or to 85% after being stretched while remaining attached (Fig. 5C). Stretched lifted cells exhibited the highest percentage of junctional plakoglobin at 98% (Fig. 5C).

Western blot analysis (relative to unstretched, unlifted cells) revealed that lifting cells resulted in a 42% decrease in the total expression level of plakoglobin (Fig. 5D, E). Cyclic stretch led to a 17% up-regulation of plakoglobin in attached cells. After lifting, cyclic stretch led to a 53% up-regulation of plakoglobin (Fig. 5D, E), or an increase of 166% when compared with the unstretched, lifted cells. Thus, for cells in the lifted region, both the spatial arrangement and expression level of plakoglobin are significantly altered, but with differences from actin. While both total actin and plakoglobin expression dropped significantly on lifting, plakoglobin remains significantly mechanoresponsive, while actin's sensitivity is considerably diminished.

Plakoglobin knockdown mechanically weakens lifted cell sheets

Due to the responsiveness of plakoglobin in lifted cells, RNA interference was next used to determine the role of plakoglobin in regulating sheet cohesion. Brightfield images of lifted cells acquired 4 days after plakoglobin knockdown demonstrated small tears in the cell sheets, while stretched lifted sheets exhibited significant tears (Fig. 6A). Immunofluorescence staining showed diminished plakoglobin expression in both unstretched and stretched cases. To confirm the effects of siRNA knockdown, western blot analysis of siRNA transfection was performed. Lifted cells transfected with plakoglobin siRNA displayed a 77% reduction of plakoglobin expression level relative to cells transfected with negative control siRNA (Fig. 6B, C). Stretch caused a 57% up-regulation of plakoglobin expression level.

FIG. 5. Plakoglobin and cell sheet mechanical properties are significantly altered in lifted cells. Immunofluorescence images are shown of (A) plakoglobin and (B) triple staining for plakoglobin, actin, and nuclei in unstretched control (top left), unstretched partial-lifted (top right), stretched control (bottom left) and stretched, partially-lifted (bottom right) cells. Partially lifted sheets were subjected to cyclic stretch for 4 h at 5% strain and 1 Hz before staining. Red: plakoglobin (PG), Green: actin, Blue: nucleus. Scale bar: 10 μ m. (C) Quantification of junctional plakoglobin reveals elevated junctional accumulation in both lifted and stretched cells. (D, E) Western blot analysis showed a significant reduction of total cellular plakoglobin when cell–substrate adhesion was lost, while cyclic stretch caused substantial up-regulation of plakoglobin, especially in the lifted cells. * $p < 0.05$, ** $p < 0.01$. Color images available online at www.liebertpub.com/tea

Interestingly, even with plakoglobin knockdown, stretch induced a nearly threefold increase in plakoglobin expression.

Next, tensile tests and shear tests were performed to evaluate the mechanical strength and cohesion of the siRNA transfected sheets. Mechanical tests were performed on unstretched lifted sheets with plakoglobin siRNA knockdown and compared with matched untreated lifted sheets. Similar to actin disruption, plakoglobin siRNA knockdown led to more fragile sheets (a 96% reduction in maximal strain, Fig. 6D, and an 87% reduction in sheet cohesion, Fig. 6E). The tears in mechanically conditioned sheets precluded tensile testing or cohesion measurements. These results not

only further confirm the importance of junctional plakoglobin in maintaining sheet mechanical properties, but also provide support for the notion that mechanical forces directly regulate cell–cell junctional dynamics.

Discussion

In this study, we developed a novel method to generate partially lifted substrate-free cell sheets that can be manipulated and assayed. Experimentation on these partially lifted cell sheets may provide critical information on cell–cell interactions, which have numerous downstream applications in tissue engineering, investigation of certain genetic and

FIG. 6. Plakoglobin RNA interference weakens mechanical cohesion of the substrate-free sheets. **(A)** Bright field and immunofluorescent images of plakoglobin, and fluorescent images of actin in unstretched control (top left), unstretched plakoglobin siRNA knockdown (top right), stretched control (bottom left), and stretched PG siRNA knockdown (bottom right) in lifted cells. Red: PG, green: actin, blue: nucleus. Scale bar: 10 μ m. **(B)** Western blot analysis of plakoglobin 4 days after transfection (PG siRNA KD). GAPDH was used as a loading control. **(C)** Quantification of relative plakoglobin level in each western blot lane, with values normalized to the untreated control. Western blot analysis showed a significant reduction of total cellular plakoglobin with siRNA knockdown. Stretch significantly increased plakoglobin expression levels compared with their respective unstretched controls. Both **(D)** tensile test measuring ultimate broken strain and **(E)** shear test measuring cell-cell adhesion strength showed that siRNA knockdown of plakoglobin significantly weakened the lifted sheets' cohesion. * $p < 0.05$ and ** $p < 0.01$ compared with the matched untreated control. Color images available online at www.liebertpub.com/tea

autoimmune diseases, as well as embryonic development and cell and tissue mechanics. This study focused on the characterization of cell-cell junctions in cell sheets with very limited ECM support.

We demonstrated that when cell-cell interactions dominate, the basic physiology of cells is significantly altered. Intercellular junctions may be mechanically sensitive signaling hubs that participate in mechanotransduction.^{38,39} Plakoglobin was chosen for examination, primarily because it can bind to both desmosomal and classical cadherins, and may be a mediator of crosstalk between neighboring cells.¹⁷ Our data are consistent with the notion that the cell's ability to convert physical perturbations into signaling cascades is based on the integrity of the junctional-cytoskeletal linkages.^{18,33–36} Moreover, stretch is a well-known potent stimulus for many cell functions.^{36,38} On stretching, applied physical force may stiffen the intercellular junctional-cytoskeletal network through mechanical strain-stiffening^{40,41} or signaling-

mediated reinforcement.^{16,42} Therefore, force-induced junctional assembly and accumulation can serve as a self-protective mechanism and dissipate mechanical stress across intercellular junctions/cytoskeletal networks more effectively. We hypothesize that when cell-cell interactions dominate and are subject to stretch stimuli, increased tension at intercellular junctions leads to the recruitment of junctional components, leading to further enhancement of junctional tension in a positive mechanosensory feedback loop.^{43,44} Previously characterized elevated intercellular contraction, junctional reinforcement, and increased cohesion are consistent with this hypothesis.^{14,18}

For tissue-engineering purposes, this study demonstrates that it is possible to mechanically precondition dissociated cell sheets, although not by compression. If the monolayer is intended for transplantation (by patching, for example) into tissues, then such preconditioning may alleviate the fragilities that are associated with dissociation from the

monolayer.^{2,10,45,46} As far as we know, the partial-lift method represents the first technique for achieving this goal, as current work is primarily limited to simple, short-term manipulations.²¹ We additionally demonstrate that since the lifted cells exhibit vastly different physiologies, in terms of total cellular content of actin and plakoglobin (and likely other proteins), prelifting the cells may offer an opportunity for the cells establish a new, different distribution of junctional proteins, before use. We offer the partial lift method as one that achieves these advantages by enabling the cell sheet to remain attached at edges to neighboring cells, which maintains a cellular environment which is friendly to the lifted cells, prevents significant contraction associated with full dissociation, and permits a variety of chemical and mechanical manipulation, although obtaining mechanical readouts, such as via optical tweezers or atomic force microscopy, is challenging. This method is also suited for studies of cellular physiology for development,^{6,7} where substrates play a reduced role during key junctures, as well as diseases where cell–cell interactions are altered.^{47,48} Thus, this study provides a method to engineer tissues by mimicking environments in which cell–cell interactions need to be tuned or tested.

Cell sheet engineering offers a method for reconstructing well-organized cell-dense tissues *in vitro*, while avoiding some potential scaffold-associated limitations compared with other cell sheet approaches. For example, modified thermo-responsive polymeric surfaces^{2–4} are expensive to purchase (or labor intensive to prepare) and can have a long harvesting time (>1 h). Another approach uses magnetic beads⁴⁹; however, this technique requires a peptide-conjugated magnetite cationic liposome coating, a magnetic field, and potential dose-dependent detrimental effects on cell proliferation and metabolism.⁵⁰ Electrochemical desorption of self-assembled monolayers of alkanethiol can detach cell sheets of various thickness; however, the desorbed alkanethiol molecules may remain bound to the cell membrane proteins of the detached cells, potentially leading to downstream signaling changes.⁵¹

We present a cell sheet harvesting technique using dispase, a rapid and effective protease that selectively cleaves the basement membrane zone region while preserving the viability of the cells.⁵² The amount of ECM in the cell sheet is minimal and is lower compared with some other treatments. Specifically, dispase treatment removes the lamina densa (rich in type IV collagen), but preserves type VII collagen-containing anchoring fibrils.^{52–54} However, a study using collagenase treatment showed that the fibronectin matrix of human periodontal ligament cells was maintained in a cell sheet after 2 h.⁵⁵ Finally, Lim *et al.* reported the disruption of several ECM ultrastructures with increasing duration of dispase incubation, indicating that it may be possible to reduce the ECM even further using dispase.⁵⁶ Thus, our dispase-based partial-lift technique results in a cell sheet with minimal ECM and the advantages of being readily performed, biocompatible, and open for manipulation.

In conclusion, we show that the partial-lift method results in cells sheets that primarily engage in intercellular interactions, and demonstrate that lifted cells respond by altering their baseline behavior while remaining sensitive to mechanical conditioning.

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Disclosure Statement

There are no competing financial interests.

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