Mouse Keratinocytes Without Keratin Intermediate Filaments Demonstrate Substrate Stiffness Dependent Behaviors

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Abstract—Introduction—Traditionally thought to serve active vs. passive mechanical functions, respectively, a growing body of evidence suggests that actin microfilament and keratin intermediate filament (IF) networks, together with their associated cell–cell and cell–matrix anchoring junctions, may have a large degree of functional interdependence. Therefore, we hypothesized that the loss of keratin IFs in a knockout mouse keratinocyte model would affect the kinematics of colony formation, i.e., the spatiotemporal process by which individual cells join to form colonies and eventually a nascent epithelial sheet.

Methods—Time-lapse imaging and deformation tracking microscopy was used to observe colony formation for both wild type (WT) and keratin-deficient knockout (KO) mouse keratinocytes over 24 h. Cells were cultured under high calcium conditions on collagen-coated substrates with nominal stiffnesses of ~1.2 kPa (soft) and 24 kPa (stiff). Immunofluorescent staining of actin and selected adhesion proteins was also performed.

Results—The absence of keratin IFs markedly affected cell morphology, spread area, and cytoskeleton and adhesion protein organization on both soft and stiff substrates. Strikingly, an absence of keratin IFs also significantly reduced the ability of mouse keratinocytes to mechanically deform the soft substrate. Furthermore, KO cells formed colonies more efficiently on stiff vs. soft substrates, a behavior opposite to that observed for WT keratinocytes.

Conclusions—Collectively, these data are strongly supportive of the idea that an interdependence between actin microfilaments and keratin IFs does exist, while further suggesting that keratin IFs may represent an important and under-recognized component of keratinocyte mechanosensation and the force generation apparatus.

Keywords—Mechanosensing, Keratins, Polyacrylamide gels, Traction microscopy, Intermediate filaments, Force.

INTRODUCTION

Cells interact dynamically with the extracellular matrix (ECM) in a manner that is dependent on the physical properties of the microenvironment.⁶ These mechanical cues play an important role in driving a range of cellular behaviors, such as changes in cell morphology,^{10,22,30,48,51} migration,^{30,43,46,51} proliferation,⁴⁶ differentiation,^{9,40,46} apoptosis,⁴³ force generation,⁷ and ECM remodeling.^{7,51} The fact that these behaviors are tightly regulated by mechanical cues implies that the cells are capable of “sensing” their physical environment.

The details of how living cells sense and respond to mechanical cues present within their local environment are complex, context dependent, and not completely understood.¹⁸ It is clear, however, that substrate/ECM mechanosensation occurs at the cell/ECM interface, where integrin-rich anchoring junctions, including hemidesmosomes and focal adhesions,³⁵ couple the ECM to the cytoskeleton, specifically, through connections to intermediate filaments (IF) or actin microfilaments, respectively. Both hemidesmosomes and focal adhesions also contain a host of other important integrin-associated auxiliary proteins (e.g., vinculin) that assemble on the cytoplasmic side of the junction in a context dependent manner, where they play an essential role in modulating cytoskeletal architecture, force generation, and other cellular activities.^{18,19}

Of the principle components of the cytoskeleton (microtubules notwithstanding), actin microfilaments are perhaps the best characterized and most understood for their essential role in various active mechanical processes, including mechanosensation and force generation.^{18,28} In contrast, although they are known to be dynamically regulated,⁴⁷ IFs, such as

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keratins and vimentin, are traditionally thought to function by providing passive tensile support to the cytoskeleton that stabilizes cell shape and contributes to overall cellular mechanical function.^{12,45} However, given the genetic diversity of intermediate filaments, and in particular, the abundance of different keratin genes present in the human genome, it seems reasonable to assume that specific IFs might confer functionality other than passive tensile support. Both cytoarchitectural systems—actin microfilaments and keratin IFs—are present within epidermal keratinocytes and are essential for maintaining the integrity of the human skin in states of both health and disease.³⁷ Within epidermal keratinocytes, it is reasonable to postulate that the functionalities of each of these two major cytoarchitectural system may actually have a large degree of interdependence. This assumption is based on: (1) the spatiotemporal coordination of adherens junction and desmosome assembly during nascent epithelial sheet formation¹³; (2) the tight regulation during wound healing of assembly and reorganization of focal adhesions, adherens junctions, desmosomes and hemidesmosomes^{17,29}; and (3) the known interaction of proteins like integrins and plectin with both actin and IFs.^{14,25,27,36,41} Towards this end, the role(s) of keratin intermediate filaments in the processes of keratinocyte mechanosensation and force generation, remains unclear.

In previous work, we found that human epidermal keratinocyte morphology, migration, and colony forming behaviors were significantly different on soft (~ 1.2 kPa) polyacrylamide (PA) gels compared to stiff (~ 24 kPa) gels.⁵¹ In particular, the kinematics of keratinocyte colony formation, i.e., the spatiotemporal process by which individual cells join to form multicellular aggregates and eventually a nascent epithelial sheet, appeared to unfold in a more directed manner on soft PA gels, possibly due to cell–cell mechanical communication through the underlying substrate. Additionally, we observed enhanced staining of the β_4 integrin subunit along the perimeter of colonies forming on soft PA gels, a pattern that was absent in colonies forming on stiff PA gels. Taken together, these observations suggest that *via* $\alpha_6\beta_4$, associated hemidesmosomal proteins, keratin IFs could play a role in these observed substrate dependent behaviors. Using the same experimental methodology utilized in our previous study, we set out to test the hypothesis that due to the interdependence of the microfilament and keratin IF networks, the loss of IFs in a knockout mouse keratinocyte model would affect the kinematics of colony formation and nascent epithelial sheet formation. In this work, we show that an absence of keratin IFs significantly reduces the ability of mouse keratinocytes on soft PA gels to deform the substrate

and join into colonies, which suggests that an interdependence between actin microfilaments and keratin IFs does in fact exist, and that keratin IFs are possibly an important component of keratinocyte mechanosensation and the force generation apparatus.

MATERIAL AND METHODS

Cell Culture

The isolation, generation, and characterization of wild type (WT) and knock out (KO) mouse keratinocytes immortalized cell lines in which all type I keratin genes were deleted is described in detail elsewhere.^{20,21,24,32,42} Although all type II genes remain, the absence of type I keratins renders the type II keratins unstable. Consequently, they are degraded such that they are not detectable by Western blotting. Furthermore, other IFs, such as desmin, neurofilaments, GFAP, and vimentin, are not up-regulated as a means of compensation.

Keratinocytes were cultured on type I rat tail collagen (Invitrogen, Carlsbad, CA) coated cell culture dishes and maintained in a humidified incubator with 95%/5% air/CO₂ at 37 °C. Cells were cultured in DMEM/Ham's F12 (F9092-0.46; Biochrom Ltd., Cambourne, United Kingdom), supplemented with chelex-treated (Chelex 100 Resin, Bio-Rad, Hercules, CA) 10% fetal bovine serum (Gibco/ThermoFisher Scientific, Waltham, MA),⁴ 0.18 mM adenine (Sigma Aldrich), 0.5 mg/mL hydrocortisone (Sigma Aldrich), 2.5 μ g/mL insulin (Sigma Aldrich), 100 pM cholera toxin (Sigma Aldrich), 10 ng/mL EGF (Gibco/ThermoFisher), 2 mM glutamax (Gibco/ThermoFisher), and 1 mM sodium pyruvate (Gibco/ThermoFisher). The final concentration of Ca²⁺ in the medium was 0.06 mM (i.e., low Ca²⁺ medium). Cells between passages 23 and 29 were used for all experiments.

Polyacrylamide Gels

Polyacrylamide (PA) gels measuring approximately 100 μ m in thickness were polymerized onto the surfaces of No. 1 glass cover slips as described previously.^{30,51} Briefly, ratios of 2% acrylamide/0.25% bis-acrylamide and 7.5% acrylamide/0.3% bis-acrylamide were used to fabricate soft and stiff PA gels with nominal stiffnesses of 1.2 kPa and 24 kPa, respectively. Atomic force microscopy (AFM) measurements of the Young's modulus of human dermis range between 0.1 and 10 kPa, with median values near 1 kPa.¹ AFM measurements on rat granulation tissue were higher, ranging on average from approximately 18 kPa at 7 days to 50 kPa at 12 days.¹¹ Thus, the soft (1.2 kPa) and stiff (24 kPa) PA gels in this study are within the range of physiologic relevance by design. In

addition, 0.5 μm diameter FluoSpheres® carboxylate-modified microspheres (#F8812, Life Technologies/ThermoFisher Scientific) were embedded in the gels to enable substrate deformation tracking. Pepsin-digested type I collagen (Advanced Biomatrix, Carlsbad, CA) was crosslinked to the surface of the gels using Sulfo-SANPAH. The gels were then sterilized under UV light for 15 min and stored at 4 °C prior to use.

Time-Lapse Live Cell Imaging: Colony Formation and Substrate Deformation Tracking

Immediately prior to the start of an experiment, cells were trypsinized and suspended in the base culture medium (described above) and modified such that the calcium concentration was increased to 1.2 mM (i.e., high Ca^{2+} medium) in order to induce keratinocyte colony formation *via* the calcium switch.⁵⁰ The cells were then plated on the PA gels at a density of 16,000 cells/cm². Time-lapse live cell imaging was carried out using a Nikon Eclipse Ti inverted microscope equipped with differential interference contrast (DIC) microscopy and wide-field epifluorescence capabilities, a Perfect Focus System (PFS), and a DS-Qi1 Nikon camera. Gels were positioned inside of a micro-environmental gas chamber (H201 Gas Chamber, Okolab, Pozzuoli, Italy) positioned within a cage incubator that fits over the microscope stage. This system maintains a temperature of 37 °C and humidified air with 5% CO₂. DIC images were acquired for each gel every 5 min for 24 h with a CFI plan Apo 10X DIC objective. For each DIC image of the cell field, an epifluorescence image was also taken in order to assess the associated spatial positions of the microspheres embedded within the PA gel substrate. One field of view was obtained per gel, and each experimental condition was repeated in triplicate (i.e., $n = 3$ per group).

The ability of keratinocytes to join into colonies was determined by counting the number of single cells remaining in the field over time. The number of single cells present in the microscope field was counted manually every hour over the 24 h period of the experiment. Four experimental conditions spanning both soft and stiff PA gels and either KO or WT mouse keratinocytes were each examined in triplicate. Those four conditions are designated as KO soft, KO stiff, WT soft, and WT stiff. The effect of substrate stiffness on KO and WT keratinocytes cell morphology was assessed by measuring the surface area of single cells *via* ImageJ (National Institutes of Health, Bethesda, MD). Substrate displacement fields on soft PA gels were calculated by tracking microsphere displacements with a custom template matching digital image correlation algorithm.^{31,51} For all image sets, this algorithm

for deformation tracking microscopy (DTM) was applied to the same centrally located region, measuring 761.3 μm by 621.5 μm and containing 1049 evenly spaced nodes (i.e., locations where the substrate was tracked). All displacement tracking and visualization was done in MATLAB (MathWorks, Natick, MA). Note that substrate DTM was only conducted on experiments involving soft PA gels because substrate displacements on stiff gels were too small to track with a 10x objective, as detailed in our previous work.⁵¹ Displacements can be tracked with a higher magnification objective, but the smaller field of view impairs our ability to count and track colony formation. In addition, the measured substrate displacements were not used to solve the inverse problem of calculating traction forces due to the fact that the boundary conditions are poorly defined—some substrate displacements just within the imaging area are produced by single cells and colonies immediately outside the field view. Other limitations include extremely large soft PA gels displacements and questions about appropriate constitutive equations and material properties.³⁹

Immunofluorescence

In order to visualize the organization of the actin cytoskeleton, in a parallel set of experiments, WT and KO cells were fixed with 4% paraformaldehyde for 1 h at room temperature at the 3, 6, and 24 h time points of the time-lapse experiment. Cells were permeabilized using 0.1% Triton-X for 10 min. A 1:40 ratio of phalloidin (Alexa Fluor® 594 dye, Life Technologies/ThermoFisher Scientific) was applied to the cells for 90 min at 37 °C. The samples were then washed with phosphate buffered saline (PBS) for 20 min and mounted onto microscope slides with Prolong® Gold Antifade Mountant with DAPI (Life Technologies/ThermoFisher Scientific) in order to visualize keratinocyte nuclei.

Additional experiments were conducted to visualize cell-substrate adhesions *via* β_1 and β_4 integrin subunits and cell-cell adhesions *via* E-cadherin and desmoplakin. At 24 h, samples were fixed in 4% paraformaldehyde for 5–10 min and permeabilized for 5 min in 0.2% Triton X-100. Samples were blocked in 1% BSA for 1 h at room temperature. Cells were then incubated overnight with the following combinations of primary antibodies: (i) 1:200 guinea pig anti-desmoplakin (donated by Dr. Thomas Magin, Universität Leipzig, Germany) and 1:200 rat anti-E-cadherin monoclonal (#U3254, Sigma, Saint Louis, MO); (ii) 1:200 rat anti- β_1 integrin monoclonal (#553715, BD Pharmingen, San Jose, CA) and a 1:200 mouse anti-vinculin monoclonal (#V9131, Sigma, Saint Louis, MO); and (iii) a 1:200 dilution of rat anti-

beta 4 integrin monoclonal (#553745, BD Pharmingen, San Jose, CA) and a 1:200 dilution of mouse anti-vinculin monoclonal (#V9131, Sigma, Saint Louis, MO). Samples with primary antibodies were incubated overnight at 5–8 °C. Samples were then incubated for 1 h with corresponding secondary antibodies using the following combinations: (i) 1:800 donkey anti-guinea pig Alexa Fluor® 488 (#A-11073, Life Technologies/ThermoFisher Scientific) and 1:200 goat anti-rat Alexa Fluor® 568 (#A11077, Life Technologies/ThermoFisher Scientific); (ii and iii) 1:100 goat anti-rat Alexa Fluor® 488 (#A11006, Life Technologies/ThermoFisher Scientific) and goat anti-mouse Alexa Fluor® 568 (#A11019, Life Technologies/ThermoFisher Scientific). Samples were then washed and mounted as described earlier. Note that between each step in the process samples were washed in PBS. Immunofluorescence images were acquired using either a Nikon Ti-Eclipse inverted C2 laser scanning confocal microscope or a Nikon Ti2-E inverted A1⁺ laser scanning confocal microscope, each equipped with a Plan Apo Lambda 60x/1.40NA oil-immersion objective.

Statistical Analysis

Statistical analysis was conducted in Prism (GraphPad Software, Inc., La Jolla, CA) using a two-way analysis of variance (ANOVA) with cell genotype-substrate stiffness combination (i.e., KO soft, WT stiff) and time as co-factors, followed by *post hoc* Tukey tests. Chi square tests were also performed in order to compare the displacement histograms.

RESULTS

Comparison of WT and KO Keratinocyte Morphology, Cytoskeletal Network Organization, and Cell-Cell/Cell-ECM adhesions as a Function of Substrate Stiffness

Following the calcium switch, both WT and KO keratinocytes attached to the PA gels within an hour of plating and began migrating, making contact with neighboring cells, and forming colonies. This process unfolded in a manner that was dependent on cell genotype and substrate stiffness (Movies 1–4 in Electronic Supplementary Material). Clear morphological differences were apparent between KO stiff and WT stiff keratinocytes (Fig. 1). However, the most striking differences in morphology were for KO soft and WT soft. Here, KO keratinocytes were substantially more rounded, less spread out, and possessed smaller protrusions compared to WT keratinocytes. Instead, WT were extended and spindle-shaped, although to a lesser extent than WT stiff. Quantification of average single cell area measured up to $t = 12$ h (due to the scarcity

of remaining single cells for most conditions beyond this time point) revealed that both KO stiff and WT stiff were between 1.4 and 2.4 times larger in area than KO soft and WT soft, respectively (Table 1). In addition, on both soft and stiff PA gels, single cell area was significantly smaller ($p < 0.001$) for KO compared to WT. Differences in the morphological appearance of these cells on soft and stiff PA gels continued as these cells joined into colonies and formed epithelial sheets (see below for details on colony formation).

The organization of actin filaments in WT and KO actin was also impacted by PA gel stiffness. WT stiff gels had fully-formed, thick actin filaments that resembled stress fibers, within both single cells and cells that have become integrated into a multicellular colony (Fig. 2). WT soft also possessed thick cortical actin filaments, though these filaments were less prominent than they were on stiff PA gels. The organization of actin filaments for KO stiff was essentially indistinguishable from that of WT soft. In contrast, the actin cytoskeleton of KO soft was dispersed into cytoplasmic aggregates, even after the establishment of colonies.

Additional immunofluorescence labeling on the nature of cell-substrate adhesions at 24 h indicated that, within all four test conditions (WT soft/stiff and KO soft/stiff), vinculin and β_1 were organized into coincident punctate rectilinear streaks localized mostly to the periphery of individual cells within a given colony (Figs. 3a–3d). Some limited diffuse cytoplasmic β_1 staining was also present, but without any major discernible differences amongst the test conditions. In addition to organized rectilinear streaks, vinculin was also present in a smaller stippled pattern that often outlined the peripheral margins of apposed cells. Note that these same vinculin patterns are also observable in the β_4 /vinculin images described below (Figs. 3e–3h).

Within both KO conditions, no obvious differences in β_4 were observed. It was present largely in a disorganized cytoplasmic pattern, with some localized areas near the cell periphery containing faint stippled linear thread-like forms (Figs. 3e and 3f). For WT soft, β_4 was organized into intense punctate linear streaks (Fig. 3g). These streaks appeared to be spatially correlated but not coincident with the punctate vinculin streaks at the cell margins. A few localized regions of β_4 organization were present for WT stiff (Fig. 3h), but not nearly as prominently or to the same extent as for WT soft.

E-cadherin and desmoplakin immunofluorescence labeling also was performed in order to provide more insight on the nature of the cell-cell adhesions present at 24 h (Figs. 3i–3l). Actin-associated E-cadherin demonstrated a stippled peripheral staining pattern that did not appreciably differ amongst the four test

FIGURE 1. KO and WT cell morphology on soft and stiff substrate at $t = 3, 6$, and 24 h after plating the cells onto soft and stiff PA gels. KO soft (a, e, i), KO stiff (b, f, j), WT soft (c, g, k), and WT stiff (d, h, l) PA gels show distinct differences in morphology and colony forming behaviors. KO cells on soft PA gels were more rounded with fewer protrusions compared to WT and stiff gels, whereas the cells were more elongated and spindle-shaped (see insets a, b, c, d for higher magnification). Single cells (red circles) were still present on KO soft gels after 24 h (i), but not for the other three conditions (j, k, l).

TABLE 1. Single cell areas at selected time points up to 12 h for each experimental condition.

Condition	Cell area ($\times 10^3 \mu\text{m}^2$)				
	1 h	2 h	4 h	8 h	12 h
KO soft	4.13 ± 2.31	4.62 ± 2.19	4.72 ± 2.18	4.20 ± 2.13	5.12 ± 2.53
KO stiff	6.97 ± 4.22	6.83 ± 3.75	7.07 ± 3.33	7.99 ± 5.56	7.51 ± 5.02
WT soft	6.30 ± 3.27	7.01 ± 6.47	6.46 ± 3.09	9.05 ± 4.86	12.33 ± 6.07
WT stiff	11.29 ± 5.59	11.67 ± 6.22	11.97 ± 5.06	13.46 ± 7.02	13.38 ± 5.15

conditions. On the other hand, keratin-associated desmoplakin staining in KO soft (Fig. 3i) was nearly absent, in striking contrast to the other three test conditions. Diffuse cytoplasmic desmoplakin staining was apparent in KO stiff (Fig. 3j), and abundant desmoplakin staining was present in both WT soft and WT stiff. With regards to both WT conditions, desmoplakin was more cytoplasmic on stiff PA gels, and thus similar to KO stiff, while desmoplakin was

more peripheral at the membrane edges of apposing cells within a colony in WT soft (Fig. 3k).

Comparison of Cell-Induced Substrate Displacements Between WT and KO Keratinocytes

Both KO and WT keratinocytes exerted traction forces on the soft PA gels and produced displacement fields that increased in magnitude during the course of

FIGURE 2. Comparison of F-actin (red) organization and cell nuclei (blue) at $t = 3, 6$, and 24 h. Distinct differences in F-actin organization were apparent as a function of cell genotype and PA gel stiffness at $t = 3, 6$, and 24 h, respectively. Cortical actin could be found in KO on stiff PA gels (b, f, j) and in WT on both soft (c, g, k) and stiff (d, h, l) PA gels. In contrast, only cytoplasmic aggregates of actin were observed for KO on soft PA gels. Images (m, n, o, p) are larger fields of view of images (i, j, k, l).

the experiment as colonies formed (Fig. 4). Histograms of the displacement fields show that the magnitude of the displacements increased significantly ($p < 0.001$) over time for both KO and WT on soft PA gels, and that these displacements were significantly greater ($p < 0.001$) for WT compared to KO.

The maximum displacements in WT quickly increased and then plateaued to an average value of $101.8 \pm 4.9 \mu\text{m}$ at 14 h (Fig. 5). These values were significantly larger ($p < 0.001$) compared to KO cell-induced displacements, which increased at a slower rate to an average maximum displacement value of $64.8 \pm 3.0 \mu\text{m}$ at 17 h. The average of the top 10% of tracked substrate displacements also indicated significant differences between KO and WT (Table 2). For KO, the top 10% of displacements increased from $15.4 \pm 1.9 \mu\text{m}$ at 3 h to $48.0 \pm 12.3 \mu\text{m}$ at 24 h ($p < 0.001$). For WT, the displacements were much

higher at $21.2 \pm 6.0 \mu\text{m}$ at 3 h and 91.8 ± 9.5 at 24 h ($p < 0.001$).

The area of the field experiencing large displacements was also greater for WT compared to KO (Table 3). The average percentage of tracked locations on the gel (i.e., nodes) exceeding $30 \mu\text{m}$ of displacement was significantly higher ($p < 0.03$) for WT ($30.4\% \pm 14.5\%$) compared to KO ($2.1\% \pm 2.4\%$).

Kinematics of WT and KO Colony Formation as a Function of Substrate Stiffness

The percentage of single cells remaining in the field each hour over 24 h was used as an *ad hoc* measure of the potential for colony formation (Fig. 6). Based on this metric, the colony forming potential was greatest for WT soft, followed by WT stiff, KO stiff, and KO soft PA gels. Within the first few hours of the experi-

FIGURE 3. Comparison of cell-ECM and cell-cell adhesions at $t = 24$ h. (a–d) Integrin β_1 subunit (green) and vinculin (red). (e–h) Integrin β_4 subunit (green) and vinculin (red). (i–l) Desmoplakin (green) and E-cadherin (red).

ment, the percentage of single cells remaining in the field for WT soft decreased approximately three-fold faster compared to KO soft and two-fold faster compared to KO stiff. During this period, the percentage of single WT cells on soft PA gels also decreased 1.5-fold faster than WT on stiff PA gels.

Another *ad hoc* metric used to assess differences in the rate of colony formation was the time it took for 80% and 98% of the cells to join into a colony (Table 4). On soft PA gels it took 1.7 ± 0.6 h until 80% of the WT cells were joined into a colony. By comparison, it took 3.7 times longer (6.3 ± 3.5 h) for the KO cells to have 80% of the cells associated with a colony. It also took longer at 2.7 ± 0.6 h for 80% of WT cells on stiff PA gels to join into colonies compared to soft PA gels. In contrast, the time required for 80% of KO cells on stiff PA gels to join colonies (5.3 ± 2.1 h) was shorter on stiff PA gels compared to soft PA gels. Differences between groups, however, were not statistically significant. These trends were also reflected in the amount of time until 98% of the cells had joined into colonies (Table 4). Except in the case of KO soft, the percentage of single cells continued to decrease

over time until no single cells remained. For KO soft, however, even after 24 h $6.6\% \pm 1.9\%$ of the KO cells remained single and unassociated with a multicellular colony. Differences were significant ($p < 0.05$) between all groups except between WT soft and WT stiff. Though not significantly different, the rate of colony formation was higher for WT soft compared to WT stiff, a result consistent with our previous work involving human keratinocytes.⁵¹

DISCUSSION

The absence of keratin IFs dramatically affected cell morphology, cytoskeletal and cell adhesion protein organization, substrate displacements, and colony forming potential in a manner that was dependent on PA gel stiffness. The observation that cell spread area was greater on stiff PA gels compared to soft PA gels is consistent with our previous work,⁵¹ and it is well reported in the literature for an array of different cell types.^{2,33,34,43,49} The fact that KO cell spread area was reduced compared to WT, particularly on soft PA gels,

FIGURE 4. Displacement tracking (DTM) of KO and WT on soft PA gels at 3, 6, and 24 h. The tracked displacements are overlaid on the DIC images. Arrows show the direction and magnitude of substrate displacements. Below each image is the corresponding histogram showing the percentages of tracked substrate locations (i.e., nodes) as a function of substrate displacements. Chi-squared tests indicate that the magnitude of the displacements increases significantly with time ($p < 0.001$), and that displacements are significantly higher for WT than for KO ($p < 0.001$).

has not been previously observed, perhaps because prior work cultured these keratin IF KO cells in low calcium medium on collagen-coated tissue culture plastic substrates, which have a stiffness on the order of gigapascals, several orders of magnitude stiffer than our PA gels. In terms of cytoskeletal organization, Seltmann *et al.* found that deletion of keratin IFs had

no noticeable impact on actin (or tubulin) organization,³⁸ which along with other findings, suggested that keratin IFs provide mechanical support to the cell independent of actin or tubulin. In this study, the actin organization of KO keratinocytes on soft PA gels (~ 1.2 kPa) was demonstrably impaired, similar to what has been observed in keratin 8/18 KO hepatic

FIGURE 5. Maximum displacements produced by KO and WT on soft PA gels over time. Data are presented as the average and standard deviation of three samples for each cell genotype. A two-way ANOVA indicates that maximum displacements were significantly higher ($p < 0.001$) in WT compared to KO.

TABLE 2. Averages and standard deviations of the top 10% of substrate displacements present in the microscopic field at 3, 6, and 24 h.

	Displacement (μm)		
	3 h	6 h	24 h
KO	15.4 ± 1.9	23.6 ± 4.5	48.0 ± 12.3
WT	21.2 ± 6.0	46.8 ± 12.5	91.8 ± 9.5

TABLE 3. Percentages of nodes in the substrate displacement field exceeding $30 \mu\text{m}$ at 3, 6, and 24 h.

	Percentage of nodes		
	3 h	6 h	24 h
KO	0.0 ± 0.0	2.1 ± 2.4	36.2 ± 21.5
WT	1.8 ± 3.2	30.4 ± 14.5	69.6 ± 10.8

epithelial cells, as reported by Bordeleau *et al.*³ Actin in these cells also was less organized and did not form fibrillar networks on soft (1.8 and 3 kPa) fibronectin (FN)-coated PA gels. Actin networks did form on stiff FN-coated glass, although the actin filaments were not as prominent as for the WT hepatic cells grown on the same “stiff” substrate.

Additional immunofluorescence labeling of cell-ECM adhesions revealed no clear differences in integrin subunit β_1 expression or localization amongst the four conditions, despite the fact that actin organization was markedly different for KO soft. On the other hand, β_4 organization was affected by both cell type and gel stiffness. β_4 staining was notably more diffuse and less organized for KO soft compared to KO stiff, with more punctate staining on WT soft vs. WT stiff. These observations also are consistent both with those

FIGURE 6. Percentages of single cells remaining in the field of view over time. The decrease was faster for WT compared to KO. Single KO cells still remained on soft PA gels after 24 h. Data are presented as the average and standard deviation of three samples for each cell genotype and gel condition. Note that there is a break in the y-axis. A two-way ANOVA with time and experimental condition as co-factors, followed by Tukey *post hoc* tests, indicated significant differences ($p < 0.001$) amongst all experimental conditions except WT stiff and WT soft.

TABLE 4. Colony formation measurements based on the percentages of single cells remaining in the field.

Condition	Less than 20% of cells are singleTime (h)	Less than 2% of cells are singleTime (h)
KO soft	6.3 ± 3.5	> 24
KO stiff	5.3 ± 2.1	15.7 ± 2.1
WT soft	1.7 ± 0.6	11.0 ± 1.7
WT stiff	2.7 ± 0.6	9.7 ± 1.2

of Seltmann *et al.*, who demonstrated that keratins are required to form stable β_4 positive hemidesmosomes, and that in the absence of keratins, β_4 is more scattered and fewer hemidesmosomes exist,³⁸ and with our previous work, where similar differences in β_4 labeling were observed in human keratinocytes on soft vs. stiff PA gels.⁵¹

Although the punctate and peripheral/junctional membrane staining patterns for vinculin were present in all four conditions, some subtle differences were observed. The pattern of punctate staining appeared more prevalent in WT soft vs. WT stiff. To a lesser degree, KO soft had the appearance of slightly more peripheral membrane staining and less punctate staining compared to KO stiff. Towards this end, it is tempting to suggest that vinculin staining at cell-cell junctions on soft PA gels could reflect increased transmission of tensional forces *via* adherens junctions, particularly as vinculin recruitment to E-cadherin complexes recently has been shown to occur in

response to increased intracellular tension.¹⁵ In this context, cells within a colony evolving on a soft substrate might favor cell-cell adhesions over cell-ECM adhesions as a means of balancing the intrinsic cytoskeletal tension required for normal cell functioning.

Major differences in the generation of substrate displacements, and by inference, force generation, between KO soft and WT soft also were found. To our knowledge, this behavior has not been observed before. These data are supportive of the notion that hemidesmosomes also could play a role in traction force generation. It is also possible that these data are reflective of the abnormal formation/organization of the actin microfilament network present in the KO keratinocytes, rather than a direct reduction in force generation through the hemidesmosomes. The differences in actin organization observed here could result from altered desmosomes (as evidenced by the lack of desmoplakin in KO soft), which is consistent with the notion that desmoglein 1 can organize actin.²⁶ Similarly, Broussard *et al.* demonstrated that strengthening connections between desmosomes and IFs in an immortalized human keratinocyte cell line served to increase cell-substrate and cell-cell forces in both cell pairs and cell sheets.⁵ Disrupting desmoplakin linkages between desmosomes and the keratin IF system, i.e., weakening the mechanical junction, had the opposite effect.⁵ Changes in cell force generation were strongly correlated to actin filament organization, which in turn was modulated by desmosome-keratin linkages. A significant reduction in keratinocyte cell-substrate deformations, and hence force generation, coupled with the observations of diffuse β_4 staining and an absence of desmoplakin in KO soft, could be due to a similar mechanism. Comparable findings have been reported for vimentin, the dominant IFs in fibroblasts and endothelial cells.^{8,44,45} For example, Eckes *et al.* found that murine fibroblasts deficient in vimentin had significantly reduced ability to contract free floating collagen gels, reduced cell stiffness, and altered actin organization compared to wild type murine fibroblasts.⁸ It is reasonable to expect that vimentin and keratin IFs perform similar cytoskeletal functions and that their absence will alter the balance of cytoskeletal forces, and thus, the mechanobiology of a cell.

We also observed major differences between WT and KO keratinocytes in their ability to form colonies. WT colony formation occurred faster on soft compared to stiff PA gels, an observation in agreement with our prior work on primary human keratinocytes.⁵¹ Interestingly, this pattern of behavior reversed for the KO cells, which appeared to form colonies more quickly on stiff vs. soft substrates. Most strikingly, a small fraction of single KO cells still remained on soft PA gels at 24 h, an aspect of colony

formation that was not observed for the other three cases considered here. Furthermore, in reviewing the time-lapse image data, we observed that the majority (~ 85%) of these remaining single KO cells had been part of colony at earlier time points before detaching and becoming single again. Such behavior implies a failure to form stable desmosomes and/or adherens junctions. With respect to desmosome formation, Homberg *et al.* showed that desmosomal proteins are downregulated in our keratin KO cells, and as a result, the integrity of the epithelial sheets that formed are weakened.¹⁶ Remarkably, in the current study desmoplakin staining was largely absent at the plasma membrane in KO cells grown on soft PA gels, whereas it was localized at low levels along the plasma membrane of KO cells grown on a stiff substrate. This paucity of desmoplakin could result from an altered crosstalk between adherens junctions and desmosomes that is mediated by desmoglein 1.²⁶ As indicated by increased vinculin staining in KO cells on soft substrates, adherens junctions could be under elevated tension in the absence of keratins. This could lead to altered perijunctional actin organization which in turn might weaken desmosomes. It has been previously shown that adherens junctions and desmosomes are formed in a spatiotemporally coordinated manner.¹³ When the ability to form E-cadherin-mediated adherens junctions is diminished, desmosome formation is perturbed.²³ Likewise, increased cortical tension also could impair desmosome formation or maintenance. As such, it is reasonable to speculate that given the interdependence of the actin microfilament network on the presence of a functioning keratin IF network, so too is the function of adherens junctions interdependent on the function of desmosomes.

CONCLUSION

Live-cell time-lapse imaging and DTM was performed on WT and KO keratinocytes cultured on collagen-coated soft and stiff PA gels in order to better understand the role of keratin IFs in keratinocyte mechanosensing and force generation. KO cells demonstrated diminished spread areas, smaller substrate displacements, changes in cytoskeleton and adhesion protein organization, and an overall reduced ability to form colonies of multicellular colonies when exposed to high calcium culture conditions. These results clearly illustrate that the keratin IF cytoskeleton also plays an important role in keratinocyte mechanobiology. Our results strongly suggest that mechanosensing and force generation within keratinocytes, cellular processes traditionally thought to be inherent to only the actin microfilament network,

are also dependent on the presence of a functioning intermediate filament network within the cell.

ELECTRONIC SUPPLEMENTARY MATERIAL

The online version of this article (<https://doi.org/10.1007/s12195-018-0526-y>) contains supplementary material, which is available to authorized users.

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CONFLICT OF INTEREST

Hoda Zarkoob, Sathivel Chinnathambi, Spencer A. Halberg, John C. Selby, Thomas M. Magin, and Edward A. Sander declare that they have no conflict of interest.

ETHICAL STANDARDS

No human or animal studies or were carried out by the authors for this article.

REFERENCES

- Achterberg, V. F., *et al.* The nano-scale mechanical properties of the extracellular matrix regulate dermal fibroblast function. *J. Invest. Dermatol.* 134(7):1862–1872, 2014.
- Aghvami, M., K. Billiar, and E. A. Sander. Fiber network models predict enhanced cell mechanosensing on fibrous gels. *ASME J. Biomech. Eng.* 138(10):101006, 2016.
- Bordeleau, F., *et al.* Keratin 8/18 regulation of cell stiffness-extracellular matrix interplay through modulation of Rho-mediated actin cytoskeleton dynamics. *PLoS ONE* 7(6):e38780, 2012.
- Brennan, J. K., *et al.* Improved methods for reducing calcium and magnesium concentrations in tissue culture medium: application to studies of lymphoblast proliferation in vitro. *In Vitro* 11(6):354–360, 1975.
- Broussard, J. A., *et al.* The desmoplakin/intermediate filament linkage regulates cell mechanics. *Mol. Biol. Cell* 28:3156–3164, 2017.
- Charras, G., and E. Sahai. Physical influences of the extracellular environment on cell migration. *Nat. Rev. Mol. Cell Biol.* 15(12):813, 2014.
- Discher, D. E., P. Janmey, and Y. L. Wang. Tissue cells feel and respond to the stiffness of their substrate. *Science* 310(5751):1139–1143, 2005.
- Eckes, B., *et al.* Impaired mechanical stability, migration and contractile capacity in vimentin-deficient fibroblasts. *J. Cell Sci.* 111(13):1897–1907, 1998.
- Engler, A. J., *et al.* Matrix elasticity directs stem cell lineage specification. *Cell* 126(4):677–689, 2006.
- Georges, P. C., and P. A. Janmey. Cell type-specific response to growth on soft materials. *J. Appl. Physiol.* 98(4):1547–1553, 2005.
- Goffin, J. M., *et al.* Focal adhesion size controls tension-dependent recruitment of alpha-smooth muscle actin to stress fibers. *J. Cell Biol.* 172(2):259–268, 2006.
- Goldman, R. D., *et al.* The function of intermediate filaments in cell shape and cytoskeletal integrity. *J. Cell Biol.* 134(4):971–983, 1996.
- Green, K. J., *et al.* The relationship between intermediate filaments and microfilaments before and during the formation of desmosomes and adherens-type junctions in mouse epidermal keratinocytes. *J. Cell Biol.* 104(5):1389–1402, 1987.
- Hamill, K. J., *et al.* BPAG1e maintains keratinocyte polarity through beta4 integrin-mediated modulation of Rac1 and cofilin activities. *Mol. Biol. Cell* 20(12):2954–2962, 2009.
- Haupt, A., and N. Minc. How cells sense their own shape—mechanisms to probe cell geometry and their implications in cellular organization and function. *J. Cell Sci.* 131(6):jcs214015, 2018.
- Homberg, M., *et al.* Distinct impact of two keratin mutations causing epidermolysis bullosa simplex on keratinocyte adhesion and stiffness. *J. Invest. Dermatol.* 135(10):2437–2445, 2015.
- Hopkinson, S. B., *et al.* Focal contact and hemidesmosomal proteins in keratinocyte migration and wound repair. *Adv. Wound Care* 3(3):247–263, 2014.
- Hytönen, V. P., and B. Wehrle-Haller. Mechanosensing in cell-matrix adhesions—converting tension into chemical signals. *Exp. Cell Res.* 343(1):35–41, 2016.
- Janostiak, R., *et al.* Mechanosensors in integrin signaling: the emerging role of p130Cas. *Eur. J. Cell Biol.* 93(10–12):445–454, 2014.
- Kröger, C., *et al.* Keratins control intercellular adhesion involving PKC- α -mediated desmoplakin phosphorylation. *J. Cell Biol.* 201(5):681–692, 2013.
- Kumar, V., *et al.* A keratin scaffold regulates epidermal barrier formation, mitochondrial lipid composition, and activity. *J. Cell Biol.* 211(5):1057–1075, 2015.
- Levental, I., P. C. Georges, and P. A. Janmey. Soft biological materials and their impact on cell function. *Soft Matter* 3(3):299–306, 2007.
- Lewis, J. E., P. J. Jensen, and M. J. Wheelock. Cadherin function is required for human keratinocytes to assemble desmosomes and stratify in response to calcium. *J. Invest. Dermatol.* 102(6):870–877, 1994.
- Loschke, F., M. Homberg, and T. M. Magin. Keratin isoforms control desmosome stability and dynamics through PKC α . *J. Invest. Dermatol.* 136(1):202–213, 2016.
- Mercurio, A. M., and I. Rabinovitz. Towards a mechanistic understanding of tumor invasion—lessons from the $\alpha 6 \beta 4$ integrin. *Semin. Cancer Biol.* 11(2):129–141, 2001.
- Nekrasova, O., *et al.* Desmosomal cadherin association with Tctex-1 and cortactin-Arp2/3 drives perijunctional

- actin polymerization to promote keratinocyte delamination. *Nat. Commun.* 9(1):1053, 2018.
- ²⁷Ozawa, T., *et al.* Dynamic relationship of focal contacts and hemidesmosome protein complexes in live cells. *J. Investig. Dermatol.* 130(6):1624–1635, 2010.
 - ²⁸Parsons, J. T., A. R. Horwitz, and M. A. Schwartz. Cell adhesion: integrating cytoskeletal dynamics and cellular tension. *Nat. Rev. Mol. Cell Biol.* 11(9):633, 2010.
 - ²⁹Pastar, I., *et al.* Epithelialization in wound healing: a comprehensive review. *Adv. Wound Care* 3(7):445–464, 2014.
 - ³⁰Pelham, Jr., R. J., and Y. Wang. Cell locomotion and focal adhesions are regulated by substrate flexibility. *Proc. Natl. Acad. Sci. USA* 94(25):13661–13665, 1997.
 - ³¹Raghupathy, R., *et al.* Identification of regional mechanical anisotropy in soft tissue analogs. *J. Biomech. Eng.* 133(9):091011, 2011.
 - ³²Ramms, L., *et al.* Keratins as the main component for the mechanical integrity of keratinocytes. *Proc. Natl. Acad. Sci. USA* 110(46):18513–18518, 2013.
 - ³³Rudnicki, M. S., *et al.* Nonlinear strain stiffening is not sufficient to explain how far cells can feel on fibrous protein gels. *Biophys. J.* 105(1):11–20, 2013.
 - ³⁴Saha, K., *et al.* Substrate modulus directs neural stem cell behavior. *Biophys. J.* 95(9):4426–4438, 2008.
 - ³⁵Schwartz, M. A. Integrins and extracellular matrix in mechanotransduction. *Cold Spring Harb. Perspect. Biol.* 2(12):a005066, 2010.
 - ³⁶Sehgal, B. U., *et al.* Integrin beta4 regulates migratory behavior of keratinocytes by determining laminin-332 organization. *J. Biol. Chem.* 281(46):35487–35498, 2006.
 - ³⁷Selby, J.C., Mechanobiology of epidermal keratinocytes: desmosomes, hemidesmosomes, keratin intermediate filaments, and blistering skin diseases. In: *Mechanobiology of Cell-Cell and Cell-Matrix Interactions*. New York: Springer, pp. 169–210, 2011.
 - ³⁸Seltmann, K., *et al.* Keratins significantly contribute to cell stiffness and impact invasive behavior. *Proc. Natl. Acad. Sci. USA* 110(46):18507–18512, 2013.
 - ³⁹Tang, X., *et al.* A novel cell traction force microscopy to study multi-cellular system. *PLoS Comput. Biol.* 10(6):e1003631, 2014.
 - ⁴⁰Trappmann, B., *et al.* Extracellular-matrix tethering regulates stem-cell fate. *Nat. Mater.* 11(7):642–649, 2012.
 - ⁴¹Tsuruta, D., *et al.* Hemidesmosomes and focal contact proteins: Functions and cross-talk in keratinocytes, bullous diseases and wound healing. *J. Dermatol. Sci.* 62(1):1–7, 2011.
 - ⁴²Vijayaraj, P., *et al.* Keratins regulate protein biosynthesis through localization of GLUT1 and -3 upstream of AMP kinase and Raptor. *J. Cell Biol.* 187(2):175–184, 2009.
 - ⁴³Wang, H. B., M. Dembo, and Y. L. Wang. Substrate flexibility regulates growth and apoptosis of normal but not transformed cells. *Am. J. Physiol. Cell Physiol.* 279(5):C1345–C1350, 2000.
 - ⁴⁴Wang, N., and D. Stamenovic. Contribution of intermediate filaments to cell stiffness, stiffening, and growth. *Am. J. Physiol. Cell Physiol.* 279(1):C188–C194, 2000.
 - ⁴⁵Wang, N., and D. Stamenovic. Mechanics of vimentin intermediate filaments. *J. Muscle Res. Cell Motil.* 23(5–6):535–540, 2002.
 - ⁴⁶Wang, Y., *et al.* Substrate stiffness regulates the proliferation, migration, and differentiation of epidermal cells. *Burns* 38(3):414–420, 2012.
 - ⁴⁷Windoffer, R., *et al.* Cytoskeleton in motion: the dynamics of keratin intermediate filaments in epithelia. *J. Cell Biol.* 194(5):669–678, 2011.
 - ⁴⁸Yeung, T., *et al.* Effects of substrate stiffness on cell morphology, cytoskeletal structure, and adhesion. *Cell Motil. Cytoskelet.* 60(1):24–34, 2005.
 - ⁴⁹Yip, C. Y., *et al.* Calcification by valve interstitial cells is regulated by the stiffness of the extracellular matrix. *Arterioscler. Thromb. Vasc. Biol.* 29(6):936–942, 2009.
 - ⁵⁰Zamansky, G. B., U. Nguyen, and I. N. Chou. An immunofluorescence study of the calcium-induced coordinated reorganization of microfilaments, keratin intermediate filaments, and microtubules in cultured human epidermal keratinocytes. *J. Investig. Dermatol.* 97(6):985–994, 1991.
 - ⁵¹Zarkoob, H., *et al.* Substrate stiffness affects human keratinocyte colony formation. *Cell. Mol. Bioeng.* 8(1):32–50, 2015.