

Final Draft
of the original manuscript:

Nie, Y.; Xu, X.; Wang, W.; Ma, N.; Lendlein, A.:

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In: Clinical Hemorheology and Microcirculation. Vol. 76 (2020) 2, 329 - 340. First published online by IOS Press: 08.09.2020

DOI: 10.3233/CH-209217

<https://dx.doi.org/10.3233/CH-209217>

Spheroid formation of human keratinocyte: balancing between cell-substrate and cell-cell interaction

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Abstract

BACKGROUND: The formation of spheroids is tightly regulated by intrinsic cell-cell and cell-substrate interactions.

OBJECTIVE: The chitosan (CS)-coating was applied to investigate the driven force directed the spheroid formation.

METHODS: The effects of CS on cell functions were studied. Atomic force microscopy was employed to measure the cell- biomaterial interplay at single cell level.

RESULTS: HaCaT cells shifted from their flattened sheet to a compact 3D spheroidal morphology when increasing CS-coating concentration. The proliferative capacity of HaCaT was preserved in the spheroid. The expression and activation of integrin $\beta 1$ (ITGB1) were enhanced on CS modified surfaces, while the active to total ratio of ITGB1 was decreased. The adhesive force of a single HaCaT cell to the tissue culture plate (TCP) was 4.84 ± 0.72 nN. It decreased on CS-coated surfaces as CS concentration increased, from 2.16 ± 0.26 nN to 0.96 ± 0.17 nN. The adhesive force between the single HaCaT cell to its neighbor cell increased as CS concentration increased, from 1.15 ± 0.09 nN to 2.60 ± 0.51 nN.

CONCLUSIONS: Conclusively, the decreased cell- substrate adhesion was the main driven force in the spheroid formation. This finding might serve as a design criterion for biomaterials facilitating the formation of epithelial spheroids.

Keywords: HaCaT cells, spheroid formation, atomic force microscope, single-cell adhesion, chitosan

Introduction

In native solid tissue, different cells are assembled in a complex three-dimensional (3D) environment, which is very different from the conventional monolayer culture system [1,2]. Monolayer cultures of tissue cells typically are lacking biochemical and biophysical signals related to the 3D character of a microenvironment. Since monolayer cells adhered to the non-physiologic culture substrates, alterations in cell-cell and cell-matrix interactions are able to induce cell malfunction [3]. In particular, conventional cell cultures cannot simulate the complexity and heterogeneity with a tissue-specific organization and architecture [4].

A 3D culture could represent living tissue outside the body in an accurate manner and to a certain extent avoid large-scale and cost-intensive animal testing [3,5-7]. Spheroid cultures of cells, as a promising 3D culture system, mimics in vivo native tissue more accurately than monolayer cultures [4,8]. There are various advantages of spheroid cultures in comparison with other 3D systems, including its simplicity, robustness, and high efficiency [9,10]. Further, spheroid cultures are compliant with high throughput screening systems and scalable in different culture platforms [11]. Skin spheroids have recently emerged as an essential tool in the areas of drug development, drug and toxicology screening, and skin reconstitution as well as disease modeling [4,12]. In spheroid culture, the dissociated skin progenitor cells underwent stepwise morphological transitions similar to the morphogenesis process of native skin development, including aggregation, polarization as well as coalescence, and could at the end form the hair-bearing skin [13]. In addition, the spatial arrangement of these keratinocytes when grown as spheroids exhibited the best physiology and functionality [14]. With these advantages, a variety of different methods for the formation of multicellular spheroids has been developed over the past years. However, it is still unclear how to assemble the spheroid in a more efficient manner. The desired biophysical and mechanical conditions for the spheroid formation need to be elucidated.

Understanding the interactions at the cell-substrate interface is important to elucidate the mechanistic of the self-assembly of cells into multicellular spheroids, organs, and tissues [15,16]. Among these interactions, adhesion of cells to one another and to the substrates is one of the key regulators that modulates the cell assembly [8]. Typical cell adhesion assays, such as the flow chamber assay, are not able to quantify the cell adhesion strength. Thus, methods that can directly measure and precisely quantify the adhesive forces between cells and substrates are necessary. New approaches including magnetic tweezers, micropipette, and

atomic force microscope (AFM) provide versatile tools to quantify the cell-substrate interaction [17].

In general, the spheroid is formed when cell-cell interaction is favored over cell-substrate interactions [18]. Chitosan (CS) has been widely studied as a non-adherent polymer to promote the formation of cell spheroids [19,20]. Cell adhesion was proportional to the charge density and the smoothness of CS surface [21]. Cell adhesion on the CS/poly(ϵ -caprolactone) (PCL)-blended biomaterials could be controlled though altering the CS proportion [22], in which CS induced the spheroid formation and PCL supported the adhesion and spreading of cells [22,23]. In our study, we hypothesized that the cell-cell and cell-substrate adhesion could be controlled through surface modification of the culture substrates, which led to the spheroid formation of keratinocyte. CS was applied in different concentrations as a coating of the cell culture substrates to study the interactions at the cell-substrate interface. To precisely evaluate the force, AFM-based single-cell force spectroscopy was used to quantify the adhesion of a living cell to its neighbor cell and to the culture substrates under physiological conditions. The expression and activation adhesion molecule and the proliferative capacity of HaCaT were also examined. The present study explored the interactions at the cell-substrate interface at single cell level and provided quantitative interpretation of cell spheroid formation, which was achieved through coating the culture substrates with CS. Decreased cell-substrate adhesion and increased cell-cell interaction were the driven force of spheroid formation on CS-coated surfaces. This finding could provide more insight into materials design for the control over cell self-assembly.

Method

Preparation of chitosan substrate

Chitosan ($M_w=100.000-300.000 \text{ g}\cdot\text{mol}^{-1}$, Acros Organics, Belgium) was dissolved in the presence of 0.5M hydrochloric acid (HCl) to prepare a 1.0% w/v stocking solution. To obtain 0.0625, 0.125, 0.25, and 0.50% (w/v) chitosan solution, different volumes of distilled water were added to 1.00% w/v chitosan stocking solutions. To prepare the chitosan-coated substrates, 1 mL chitosan solutions were used to coat 10 cm² tissue culture polystyrene (TCP) plates (Falcon, Thermo Fisher Scientific Inc., Germany) and incubated at 37 °C for 24 h. After drying, the formed chitosan thin membrane substrates were washed, and HCl was neutralized in PBS buffer (Life Technologies, Germany).

Characterization of CS-coated surfaces

Adsorption of chitosan was quantified using fluorescamine assay [24]. Briefly, CS-coated substrates were washed with 1mL PBS buffer (Life Technologies, Germany) for three times and the elute was collected. To measure the amount of CS in the elute, 100 μ L elute was mixed with 10 μ L fluorescamine (2 mg/mL, Sigma-Aldrich, Germany). Fluorescamine reacts with the amine group of CS, which results in fluorescent products. The fluorescence intensity was detected at 390 nm excitation and 515 nm emission using a microplate reader (Infinite 200 PRO, Tecan Group Ltd., Switzerland). A standard curve representing the relationship between the CS concentration and the fluorescence intensity was created. The amount of CS adsorbed to the substrates was acquired by the difference between the initial amount of CS for coating and the amount of CS in the elute. The topography of CS-coated surface was determined using an atomic force microscope (Nanowizard4, JPK, Germany) equipped with a tetrahedral tip (OPUS-240AC-NG, NanoAndMore GmbH, Germany) in PBS buffer (Life Technologies, Germany). Images were acquired at a scan rate of 1 Hz and a pixel resolution of 512×512 . Data were processed using JPKSM Data Processing software (JPK, Germany).

Cell culture

HaCaT cells were purchased from American Type Culture Collection (ATCC, USA). To maintain the undifferentiated state, HaCaT cells were cultured in Keratinocyte Calcium-Free/Serum-Free Growth Medium (Sigma-Aldrich, Germany) at 37 °C in a humidified atmosphere containing 95% (v/v) air and 5% (v/v) CO₂. Once reaching 80% confluence, cells were dissociated into single cells using 0.25% (w/v) Trypsin-EDTA (Gibco, Germany) and seeded onto substrates with designed density. To initiate the spontaneous differentiation, HaCaT were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (Life Technologies, Germany) and 1 mM calcium. For morphological analysis, phase-contrast images of cells were captured using an (IX81 motorized inverted microscope (Olympus, Germany).

Adhesive force measurement

All experiments were performed at 37 ± 0.1 °C by JPK-AFM system with the CellHesion module (CellHesion[®] 200, JPK, Germany). A single HaCaT cell was captured by a tip-less AFM cantilever (ARROW-TL2Au, NanoAndMore GmbH, Germany). Cell morphology was observed by a phase-contrast microscope (Axio Observer 200, Zeiss, Germany). The cantilever bound cell was in contact with the target surface for 3 seconds followed by retracting the cell from the substrate surfaces or cell surfaces. The piezo movement as well as the deflection of the cantilever were recorded via JPK SPMControl software (JPK, Germany). The

cell-substrate adhesion was measured during the cantilever bound cell separating from the culture substrates, and the cell-cell adhesion was measured during the cantilever bound cell separating from HaCaT cells cultured on substrates with or without CS modification, respectively. Force curves were acquired and the adhesion was calculated using JPKSM Data Processing software (JPK, Germany).

Quantification of cell number

The cell number was determined based on the DNA content using the FluoReporter™ Blue Fluorometric dsDNA Quantitation Kit (ThermoFisher Scientific, Germany). The standard curve that represents the relationship between the fluorescent intensity and the cell number was obtained according to the manufacture's instruction. To quantify the number of cells on the substrates, samples were harvested at designated time points and then were frozen at -80 °C. The frozen cells were thawed and the fluorescent probes were added to the cell lysis. The fluorescence intensity was measured by microplate readers using excitation and emission filters centered at 360 nm and 460 nm (Infinite 200 PRO, Tecan Group Ltd., Switzerland). The cell number was interpolated from the standard curve.

Quantification of protein expression

In-Cell Western assay was applied to quantify protein expression level [25,26]. Briefly, cells were fixed with 4% (w/v) paraformaldehyde (Sigma Aldrich, Germany) for 10 minutes and then permeabilized with 0.1% (v/v) TritonX-100 (Sigma Aldrich, Germany) for 10 minutes at room temperature. After fixation and permeation, cells were incubated at 4 °C overnight with primary antibodies including total ITGB1 (Sigma Aldrich, Germany), and active ITGB1 (Sigma Aldrich, Germany). Secondary antibody staining (IRDye 680LT secondary antibodies, LI-COR, Germany) was performed at room temperature for 1 h. Total protein was used to normalize, which were quantified using the IRDye 800CW NHS Ester Kit (LI-COR, Germany). Following this, the absorbance at 680 nm and 800 nm were measured with Odyssey Imaging Systems (LI-COR, Germany). Fluorescent intensity was analyzed using Image J software (National Institutes of Health, USA).

Expression of keratinocyte markers

HaCaT cells were harvested and dissociated into single-cell suspensions on day 7. Then the cells were fixed with 4% (w/v) paraformaldehyde (Sigma Aldrich, Germany) for 10 minutes followed by permeation with 0.1% (v/v) TritonX-100 (Sigma Aldrich, Germany) for 10 minutes at room temperature. After cells stained with p63 (Cell Signaling Technology, Germany) and keratin (K14, Abcam,

Germany), the expression of keratinocyte markers was measured using a flow cytometer (MACSQuant[®], Miltenyi Biotec, Germany). Data were analyzed with FlowJo V10 (FlowJo LLC, USA).

Statistical analysis and error consideration

Data presented were from at least three independent experiments and data are expressed as the mean value \pm standard deviation (SD). Statistical analysis was carried out using one-way or two-way ANOVA followed by Tukey's test in GraphPad Prism software (Version 8.0.1, USA). Differences were considered statistically significant at $p < 0.05$. For the measurement of the surface topography, the noise level of the cantilever deflection detection system is < 2 pm root mean square (RMS). The sensor noise level of the AFM scanner unit is < 0.09 nm RMS in the xy-direction and < 0.04 nm RMS in the z-direction. For the single-cell force spectroscopy, the tipless AFM cantilever was calibrated three times using the thermal noise to eliminate errors [27]. Cell sliding (the lateral instability) can be avoided through control the force set point.

Results and Discussion

Characterization of the CS-coated surfaces

As the concentration of CS increased, the amount of CS coated on the substrates increased (**Figure 1A**). The topography of the uncoated bare TCP and CS-coated TCP surfaces were determined and shown in **Figure 1B**. The AFM height image showed the randomly distributed line structures on the uncoated TCP surface. Less line structures and more CS aggregates were observed as the CS solution concentration increased, suggesting CS altered the surface topography of the substrates. These aggregates might be formed during the neutralization, as the solubility of CS decreased in the neutral solutions [28]. They reflected the inhomogeneity of the coated surfaces and suggested that the surface might not be fully covered by CS. When increased CS concentration to 1.00% (w/v), porous network structures appeared and connected CS aggregates, suggesting the increased amount of CS could cover the whole substrate.

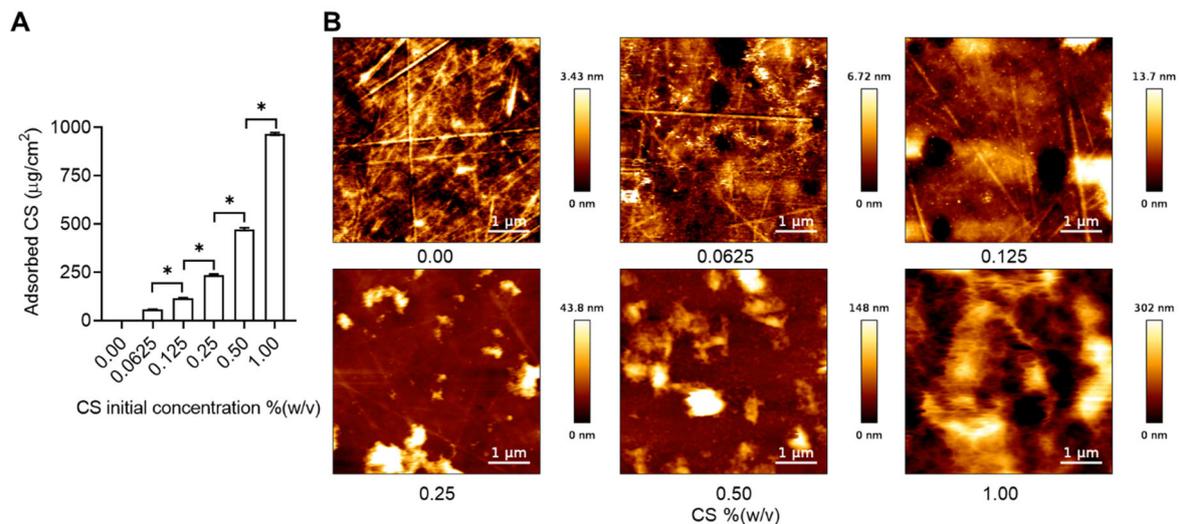


Figure 1. Characterization of CS-coated surfaces. (A) Quantification of CS adsorbed onto TCP surfaces according to the CS initial concentration (one-way ANOVA followed by Tukey's test, $n = 6$, $* p < 0.05$). (B) Surface topography of CS-coated surfaces. Representative AFM height images (Scale bar = 1 μm).

Spheroid formation of HaCaT cultured on CS-coated surfaces

A variety of methods have been applied to create 3D cell aggregates or spheroids. The formation of a multicellular spheroid depends not only on mechanical cell-cell and cell-substrate interactions but also on active cellular behavior, such as adhesion and migration [29]. In our study, we explored, whether CS would promote the spheroid formation of HaCaT. The morphology of HaCaT on different surfaces on Day 3 is shown in **Figure 2**. HaCaT cultured on TCP without coating showed a flat and fully spread morphology, while significant alterations in cell morphology were observed in the culture of HaCaT on CS treated surfaces. Compared to the cobblestone pattern of HaCaT grown as a monolayer on TCP, the HaCaT aggregates bulged and became cubical in shape on the CS-modified substrates. On 0.50% (w/v) CS-coated TCP, HaCaT aggregates were formed. With the increase in CS concentration to 1.0% (w/v), HaCaT spheroids could be observed, while they were not completely detached from the culture substrate on day 3. Our result suggested that HaCaT spheroids were not directly formed in cell suspension. Instead, they were temporarily attached to the CS surface and subsequently released from the surface over time. The appearance of CS component switched the surface from cell adhesive to non-adhesive. As a result, the cell-substrate adhesion was hindered, the balance between cell-substrate and cell-cell interaction might be disturbed and attributed to the formation of cell spheroids.

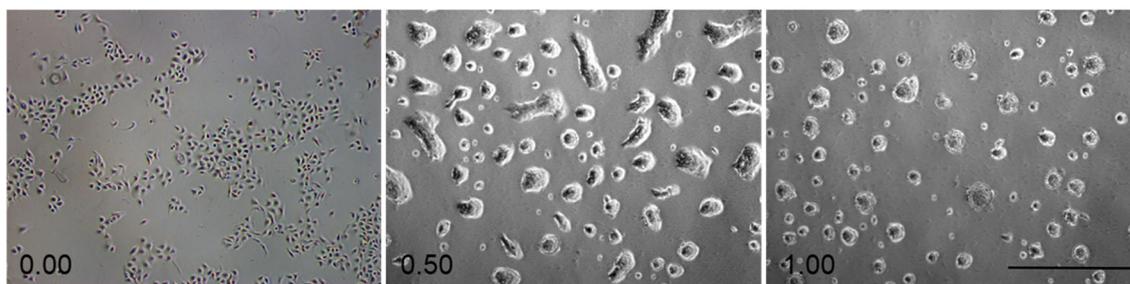


Figure 2. Morphological changes of HaCaT aggregates cultured on CS-coated surfaces. Phase-contrast images of HaCaT were taken 3 days after seeding. Significant changes in cell morphology and spheroid formation by HaCaT on CS-coated TCP were observed. (Scale bar = 200 μ m; numbers give CS concentration (%w/v)).

HaCaT cell-substrate and cell-cell adhesion

The balance of adhesion of a cell to its neighbor cells and to the extracellular matrix is the base for the formation of multicellular organs and the assembly of 3D tissues [8]. The interplay between cells and their extracellular microenvironments can be categorized into cell-cell and cell-substrate interactions [4,8]. Recent advances in biophysical measurements have provided the possibility to identify the mechanical mechanisms underlying the formation of multicellular structures. Atomic force microscopy (AFM)-based single-cell force spectroscopy could be used to detect the cell adhesion on the level of single cells under physiological conditions [30]. In addition, AFM provides precise spatio-temporal control over the interactions. To determine the adhesive force on different materials, a living single HaCaT cell was captured and firmly attached to the tip-less cantilever. Phase-contrast image of this cantilever bound cell is shown in **Figure 3A**. The entire procedure for detecting the adhesion is summarized in **Figure 3B**. The movement of the cantilever bound cell in Z direction was 70 μ m to guarantee a complete separation between the cell and the surface. In addition, it was crucial to control the contact time that determined the recognition and bond formation between the cellular adhesive molecule and its ligand. As HaCaT showed a strong propensity to adhere to TCP, a longer contacted time (5s) would lead to the detachment of the cell from the cantilever. Thus, in the force spectroscopy experiments, the contact time period was set as 3 s to ensure a complete separation. The adhesive force between the cell and the surface was defined as the maximum detachment force needed to detach the cell from the surface (**Figure 3C**). The adhesive force was detected when the cell was separated from the contacted surfaces, and the cell-cell adhesion was identified when the cell was detached from the HaCaT monolayer layer.

The cell-substrate and cell-cell adhesion were detected, respectively. There was a sharp decrease in the adhesive force between HaCaT and CS modified surfaces, as compared to those between HaCaT on non-coated TCP (**Figure 3D**). The adhesive force between the cell and CS modified surface was highest when CS concentration was 0.125% (w/v). Then the force gradually decreased. The cell-cell adhesion was increased with the increase of CS concentration (**Figure 3E**). Previous studies showed that competition between cell-cell and cell-matrix adhesion determined the cell morphology [31,32]. Thus, one-to-one comparisons between cell-substrate and cell-cell adhesion were performed. The cell-substrate adhesion was significantly greater than the cell-cell adhesion in the TCP group (0.00) as well as 0.0625% (w/v) and 0.125% (w/v) CS-coated groups. The cell-substrate adhesion and the cell-cell adhesion were comparable on the 0.25% (w/v) CS modified surface. While the cell-cell adhesion surpassed the cell-substrate adhesion on 0.50% (w/v) and 1.00% (w/v) CS-coated surfaces. Consequently, it is reasonable to infer that spontaneous formation of spheroids by cell organization might be due to the adhesive forces between the cells that surpassed those between cells and the surface of the culture substrates. Similarly, for those cells that exhibited flatted morphology, the adhesive forces between the cells were smaller than the force between cells and the substrate surface.

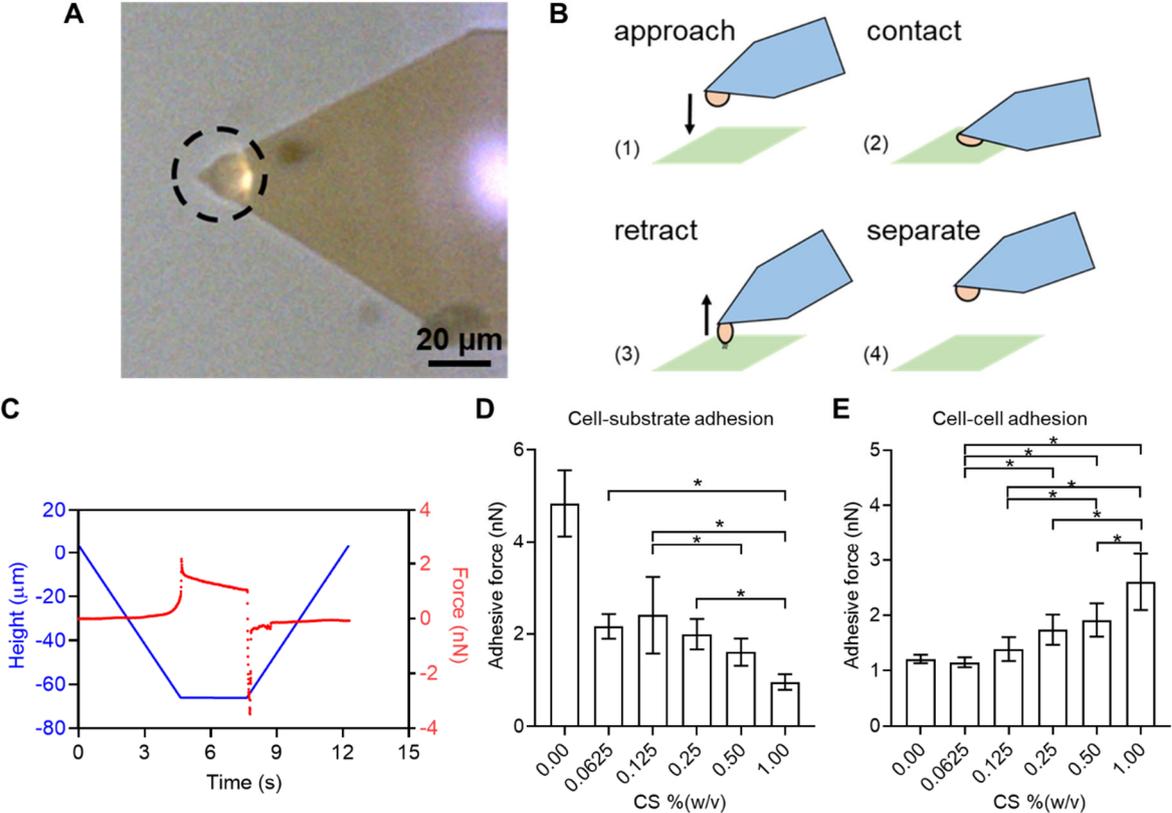


Figure 3. AFM measurement on the cell-substrate and the cell-cell interactions. (A) Living HaCaT cell mounted to a tip-less cantilever. **(B)**

Schematic of AFM-based measurement in detecting the cell-cell and cell-substrate adhesion. (1) A single HaCaT captured by cantilever at close proximity to the surface; (2) the HaCaT cell contacted to surface as it was gently pushed by the cantilever. Adhesion between the cantilever-bound cell and the target surface was formed; (3) the cantilever-bound cell started to separate from the surface as the cantilever moved vertically away from the surface; (4) a complete separation between the cell and the surface was achieved. The adhesion and the de-adhesion events were recorded and presented as a force-distance curve. (C) measurement of HaCaT adhesion. A representative z-position (height) of the cantilever-bound cell and vertical deflection (force) of the cantilever was plotted versus time. (D) Cell-substrate adhesion. Substrates were modified using CS with different concentrations. The cell-substrate adhesion was measured during the cantilever bound cell separating from the culture substrates (one-way ANOVA followed by Tukey's test, $n = 10$, significant differences among CS treated group: * $p < 0.05$). (E) Cell-cell adhesion. The cell-cell adhesion was measured during the cantilever bound cell separating from HaCaT cells cultured on substrates with or without CS modification (one-way ANOVA followed by Tukey's test, $n = 10$, significant differences among CS treated group: * $p < 0.05$).

ITGB1 expression and activation

The cell-cell and cell-substrate interactions could not only determine the external cell morphology but also could modulate the intercellular signaling molecules to modulate the organization of HaCaT architecture. Integrin family is one of the most well-known adhesion protein families, which are responsible for the cell organization on many substrate surfaces. The subpopulation of basal keratinocytes represents keratinocyte stem cells, which adhere more efficiently to the extracellular matrix (ECM) as compared to the differentiated suprabasal keratinocytes [33,34]. This phenomenon is due to the high expression level of integrin $\beta 1$ (ITGB1) in basal keratinocytes [35]. Here, HaCaT cells were maintained in a basal-like state. In order to understand the CS molecule on regulating the expression and activation of ITGB1, the total and active ITGB1 was quantified. The total ITGB1 expression was increased at the protein level on CS materials as compared to non-coated control (**Figure 4A**). The activation of ITGB1 was augmented on 0.0625% (w/v) CS-coated surface (**Figure 4B**). Of particular note, the ratio of active to total ITGB1 was decreased in HaCaT cultured on CS surfaces as compared to those on TCP (**Figure 4C**). In line with previous studies, the adhesion proteins were not directly involved in the shift of cell organization from monolayer to 3D [36]. Collectively, our data indicated that the decreased adhesive force between the cell and the culture substrates was the main driven force in the formation of HaCaT spheroids.

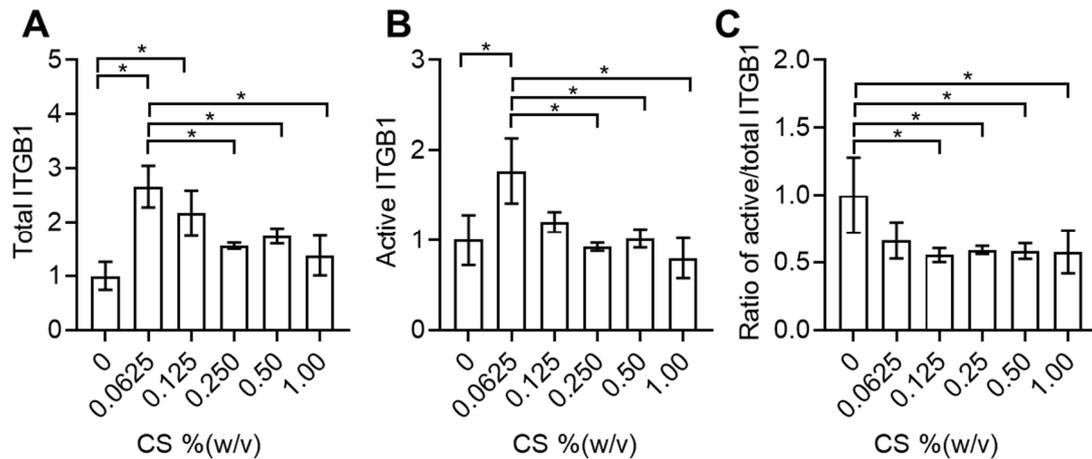


Figure 4. The expression and activation of ITGB1 in HaCaT. (A) Total ITGB1 protein expression, determined by In-Cell Western assay with total-protein normalization. (B) Active ITGB1 protein expression, determined by In-Cell Western assay with total-protein normalization. (C) Ratio of active to total ITGB1 expression level (one-way ANOVA followed by Tukey's test, $n = 3$, $* p < 0.05$).

HaCaT proliferation

As physical interactions between cells and their ECM are essential to proliferation, the proliferation capability of these cells on the CS-coated materials was explored. The proliferation assay illustrated that HaCaT cells were able to aggregate and continuously grow within the spheroids, as the cell number in CS groups was not significantly different as compared to the non-coated TCP group. HaCaT cells were seeded on different substrates for up to 7 days and cells were harvested at designed time points to determine the growth of HaCaT (**Figure 5**). With increasing the CS content on substrate surfaces, the initial cell adhesion onto the substrate surfaces was not impaired by the CS coating, as the cell number on day 0 showed no significant difference. The numbers of cells in all groups were doubled on day 3, suggesting that cell division occurred. Although, on day 3, the amount of cells in 1.0% (w/v) CS group was lower than those on TCP, significant difference was no longer observed overtime (day 7). This result suggested that cell-cell adhesion and spheroidal architecture sustained cell survival and proliferation potential. This finding is consistent with the results of studies, in which cells within the spheroid could preserve high proliferation capacity [8].

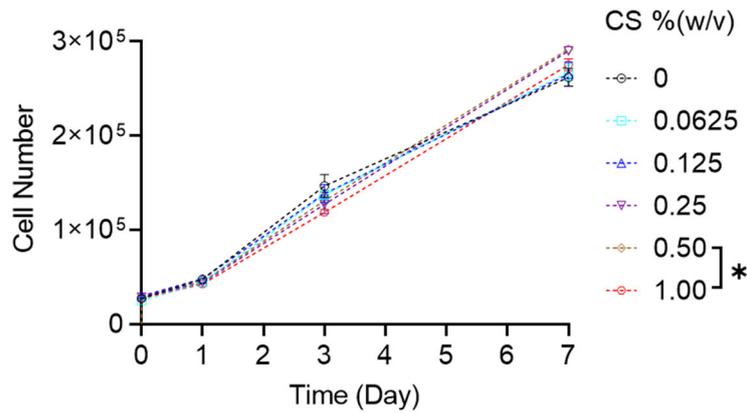


Figure 5. Proliferation potential of HaCaT cultured on CS-coated substrates. Cell proliferation assay. Cell number was quantified at designed time points (two-way ANOVA followed by Tukey's test, $n = 3$, $* p < 0.05$).

In the presence of Ca^{2+} , HaCaT cells would lose the capacity of proliferation and start spontaneous differentiation as a function of time in culture [37]. p63 and K14 are markers for the mitotically active keratinocytes of the epithelium that form the stratified surface of the skin [38-40]. Our result showed that K14 expression was similar among HaCaT cells cultured on surfaces with or without CS coating, while a higher proportion of p63-positive cells was observed in HaCaT cells cultured on CS-coated surfaces (Figure 6). Given that p63 has been identified as a specific marker for human keratinocyte stem cells [39], this result suggested that CS-mediated spheroid formation might better maintain p63 expression and thus preserve the proliferative capacity in keratinocytes.

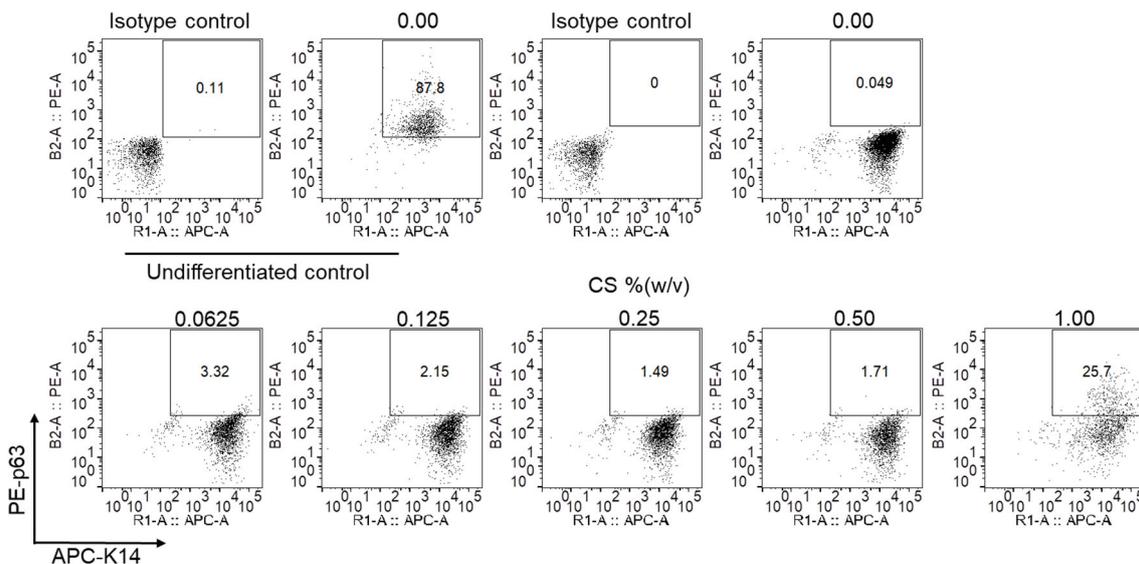


Figure 5. The expression of keratinocyte markers in HaCaT. The expression of p63 and K14 in HaCaT cells cultured on surfaces with or without CS coating was detected on day 7.

Conclusion

Studying the mechanisms of cell self-organization into spheroids will deepen our understanding of the developmental process of tissue organization and homeostasis as well as the mechanisms of various diseases. Here we applied the CS coating as a powerful tool to modulate HaCaT behaviors at the cell-substrate interface. AFM provides a powerful tool to study mechanical aspects in detecting cellular behaviors. Initially, HaCaT temporarily attached to the CS surface. Spheroids formation was mechanically facilitated when the cell-cell adhesion overrides the cell-substrate adhesion on CS coated substrates. The transition of HaCaT cells from the monolayered to the spheroidal morphology could be controlled by fine-tuning the balance between the cell-cell and cell-substrate adhesion. Physiologically, the spheroid formation in HaCaT maintained the proliferative capacity of HaCaT. Conclusively, the AFM-based single-cell force spectroscopy unraveled a role for cell-substrate and cell-cell adhesion in dictating multicellular organization of HaCaT cells. Through the application of a surface coating, the interactions and organizations of cells can be precisely controlled. This understanding serves as an initial platform to design biomaterials with appropriate biointerface to modulate cell behaviors effectively and efficiently.

Acknowledgment

This work was financially supported by the Helmholtz Association of German Research Centers through program-oriented funding, and through Helmholtz Cross Program Initiative "Technology and Medicine - Adaptive Systems", as well as by the Federal Ministry of Education and Research, Germany, through the Program Health Research (grant no. 13GW0098), and 0315696A "Poly4BioBB". Yan Nie is grateful for the support from the China Scholarship Council (CSC, No. 201706205049).

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