Regulation of pigmentation by substrate elasticity in normal human melanocytes and melanotic MNT1 human melanoma cells

Hyunjung Choi¹, Mina Kim², Song Ih Ahn², Eun-Gyung Cho¹, Tae Ryong Lee¹ and Jennifer H. Shin²,³

¹R&D Center, AmorePacific Corporation, Giheung-gu, Yongin-si, Gyonggi-do, Republic of Korea; ²Department of Mechanical Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea; ³Graduate School of Medical Science and Engineering, Korea Advanced Institute of Science and Technology, Daejeon, Republic of Korea

Abstract: The elasticity of the cellular microenvironment is a key regulator of cellular physiology in many cell types. To investigate the effects of substrate stiffness on the pigmentation process, we cultured normal human melanocytes (NHM) and MNT1 melanoma cells on laminin-coated polydimethylsiloxane (PDMS) substrates of different stiffness. The dendricity of NHM and MNT1 cells was reduced as the substrate stiffness decreased, and the degree of melanosome transfer from NHM or MNT1 cells to normal human keratinocytes was decreased on softer substrates with the reduced dendricity. Gene and protein expressions of MITF, tyrosinase, TRP2, and gp100/PMEL17 exhibited a consistent decreasing trend with the decreasing stiffness. Because the stiffness sensing is mediated by focal adhesion complex through integrin receptors, we checked laminin specific integrin alpha 6 and p-FAK for MNT1 cells to observe that the substrate adhesion was weakened as the substrate stiffness decreased. Weaker adhesion on a softer substrate was accompanied by dynamic shape changes in MNT1 cells with higher speed and larger scattering. Dendritic MNT1 cells cultured on a stiffer substrate exhibited lower migration with smaller root mean squared displacement. These results demonstrate the possibility that skin pigmentation can be influenced by mechanical properties of the cellular microenvironment and can increase when the skin becomes stiff.

Key words: mechanical stiffness – melanogenesis – melanoma – skin pigmentation

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Introduction

The pigmentation of human skin is determined by the synthesis and distribution of melanin in the epidermis (1). Melanin is a pigmented heteropolymer that is synthesized and stored in specialized cellular organelles that are referred to as melanosomes of melanocytes. The melanosomes are transported along microtubules from the perinuclear region to dendrites in melanocytes and then transferred to the neighbouring keratinocytes (2). Increases in the synthesis and distribution of melanin have been reported in human skin hyperpigmentation disorders such as melasma, postinflammatory melanoderma, and solar lentigo (3). Many physiological stimulators, such as UV radiation, inflammation, and hormones, are known to induce these hyperpigmentation disorders in melanocytes with increased expression of melanogenic genes such as tyrosinase, tyrosinase-related protein-1 and 2 (TRP-1,-2), and microphthalmia-associated transcription factor (MITF) through pathways involving a number of different soluble factors, such as alpha-melanocyte stimulating hormone (α-MSH), secreted by neighbouring cells in the skin (4).

Interestingly, skin pigmentation regions such as melanosomes and junctional nevi (JN) exhibit distinct mechanical properties and display 6-fold and 1.4-fold greater stiffness than normal surrounding skin, respectively (5). Especially, skin fibrosis with excess fibrous connective tissue, which is stiffer than surrounding healthy skin, is often accompanied by skin pigmentation (6,7). In recent years, the elasticity of the cellular microenvironment has been suggested as one of the key regulators of cellular physiology in many cell types, including fibroblasts, epithelial cells, and endothelial cells, where changes in substrate stiffness alter phenotypic traits as well as the expression levels of both genes and proteins (8). Strikingly, mesenchymal stem cells were shown to specify their lineage and commit to phenotypes with extreme sensitivity to tissue level elasticity, suggesting the responsiveness of cells to the physical state of the microenvironment resembling their physiological niche (9). Tissue stiffness was also found to actively promote malignant transformation of normal mammary gland epithelial cells to tumor cells (10). Based on these findings, it is possible to postulate that the stiffness of the cellular microenvironment can influence cellular functions and that any undesired alterations in tissue stiffness could lead to pathogenic conditions. While melanin has been shown to increase the stiffness of pigmented melanoma cells (11), the direct relevance of substrate stiffness on the pigmentation has not been determined yet. To unravel the effects of stiffness on skin hyperpigmentation, we cultured normal human melanocytes (NHMs) and MNT1 melanoma cells with or without normal human keratinocytes (NHKs) on polydimethylsiloxane (PDMS) substrates of different stiffness (E = 0.05–3.5 PPa) to study the stiffness-dependent melanosome transfer. The stiffness of these substrates was comparable to that of human skin (12). We also examined whether the changes in stiffness influenced melanogenesis-related genes and proteins. Because stiffness is known to affect cytoskeletal and adhesion proteins, we also compared the cellular morphology and motility between soft (0.05 MPa) and stiff (5.5 MPa) substrates in the...
context of pigmentation. Our results revealed that the dendricity of pigment cells and melanosome transfer from pigment cells to NHKs increased on stiffer substrates, accompanied by an increase in the gene expression of melanogenic proteins. Moreover, melanoma cells cultured on stiffer PDMS substrates migrated slowly with a much smaller root mean square displacement (RMSD) than those on softer substrates. Therefore, we suggest the possibility that skin pigmentation can be influenced by the mechanical properties of the cellular microenvironment and can increase when the skin becomes stiffer.

Methods

Cell preparation
Normal human melanocytes from the neonatal foreskin of moderately or darkly pigmented donors were purchased from Cascade Biologics (Portland, OR, USA) and cultured in Medium 254 (Cascade Biologics) supplemented with Human Melanocyte Growth Supplement (Cascade Biologics). Melanocytes at passage numbers between 3 and 4 were used for the experiments. MNT1 melanoma cells were maintained in minimum essential media (Gibco, Grand Island, NY, USA) containing 10% Dulbecco’s modified Eagle’s medium, 20 mM HEPES (Sigma, St. Louis, MO, USA), 20% fetal bovine serum, 100 U/ml penicillin G and 100 μg/ml streptomycin sulphate. MNT1 cells were incubated at 37 °C with 5% CO2 and regularly passaged at a density of 80% (1:8 ratio). Normal human keratinocytes (NHKs) were purchased from Lonza (Basel, Switzerland) and were maintained in KGM basal medium with KGM2 growth supplements containing insulin, human epidermal growth factor, bovine pituitary extract, hydrocortisone, epinephrine, transferrin and gentamicin/amphotericin B (KGM).

Elastic substrates of different stiffness

Polydimethylsiloxane substrates, a biocompatible material widely used to investigate cell behaviour (13), of varying stiffness were prepared by varying the ratio of the base agent and the cross-linker (14) using a SYLGARD 184 silicone elastomer kit (Dow Corning, Barry, UK) (See Supplementary Materials for details). Cells were plated on the 1 μg/ml laminin-coated PDMS sheet at the seeding density of 2 × 10⁵ cells per sheet (1.7 × 10⁵ cells/cm²) (See Figure S1).

Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated using TRizol™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The concentration of RNA was determined spectrophotometrically, and the integrity of the RNA was assessed using a Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). One microgram of RNA was reverse-transcribed into cDNA using SuperScript™III reverse transcriptase (Invitrogen), and aliquots were stored at −80 °C. Quantitative measurements of melanogenesis markers in each cDNA sample were performed using Assays-on-Demand™ Gene Expression Kits (Applied Biosystems, Foster City, CA, USA). The cDNA samples were analysed for tyrosinase (Hs01099965_m1), gp100/PME1L7 (Hs00173854_m1), integrin alpha 6 (Hs01041011_m1), TRP-2 (Hs01095836_m1), and MITF (Hs01117294_m1). To normalize the mRNA expression levels, the relative expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, 4333764F) was quantified. Quantitative real-time TaqMan RT-PCR technology (qRT-PCR) (Applied Biosystems) was used to determine the expression levels of selected genes. The cycling conditions included a denaturation step at 95 °C for 10 min, followed by 50 cycles of 95 °C for 15 sec and 60 °C for 1 min. The FAM fluorescence of each PCR cycle was measured using the Applied Biosystems 7500 Real-Time PCR System (Applied Biosystems). The obtained qRT-PCR data were presented as threshold cycle (Ct) values. The quantification of relative expression levels was performed using equations from a mathematical model developed by Pfaffl (15).

Western blot analysis

Normal human melanocytes and MNT1 melanoma cells cultured on substrates of different stiffness were homogenized in cell lysis buffer (RIPA buffer; Sigma) containing protease inhibitors (Sigma). Proteins were solubilized at 4 °C for 1 h. The lysate was then subjected to centrifugation at 15 000 × g for 10 min, and the supernatant was used for western blot analysis. The protein concentration of the cell lysate was determined with a Pierce BCA Protein Assay Kit (Pierce Biotechnology, Inc., Rockford, IL, USA) with bovine serum albumin as the standard. Proteins (10 μg per well) were fractionated by SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.15% Tween-20) for 1 h at room temperature and subsequently probed overnight at 4 °C with anti-tyrosinase (Upstate biotechnology, Lake Placid, NY), TRP2 (Abcam, Cambridge, MA, USA), gp100/PME1L7 (Abcam), phospho-FAK (Cell Signaling Technology (CST), Beverly, MA, USA), total FAK (CST) and anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Blots were washed three times with TBST and incubated with horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad, Hercules, CA, USA) at room temperature for 1 h. Detection was performed with the ECL system (Amer sham Pharmacia Biotech, Piscataway, NJ, USA). Relative protein levels were analysed using ImageMaster 2D Elite software (Amer sham Biosciences, Buckinghamshire, UK).

Spectrophotometric determination of the melanin content in cells

The melanin in cell lysates was separated by centrifugation at 9000 × g at 4 °C for 15 min and then dissolved in 1 N NaOH. The melanin content was measured at 490 nm in a UV-Vis spectrophotometer (Molecular devices, Sunnyvale, CA, USA).

Melanosome transfer

Normal human melanocytes or MNT1 melanoma cells and NHKs were co-cultured on PDMS substrates of different stiffnesses. Briefly, NHMs or MNT1 melanoma cells and NHKs were seeded at 0.4 × 10⁵ and 1.6 × 10⁵ (1:4 ratio) cells per sheet of PDMS, respectively, on a PDMS sheet with NHK medium. After 4 days, the cultured cells were washed three times in PBS, fixed for 15 min in 3.5% paraformaldehyde, washed again and incubated for 10 min in 0.1% Triton X-100. The cells were preincubated with 3% bovine serum albumin for 1 h at room temperature and incubated with two different primary antibodies, anti-Pmel17 antibody (1:100 dilution, Abcam, Cambridge, UK) and anticytokeratin antibody (1:100 dilution; Abcam), at 4 °C overnight. The cells were washed abundantly in PBS and incubated for 2 h with the appropriate conjugated secondary antibody. After washing, coverslips were mounted on glass slides and viewed with a LSM510 confocal laser scanning microscope (Carl Zeiss Microimaging Inc., Thornwood, NY, USA). The stained specimens were observed using an image analysis system (Dp Manager 2.1; Olympus Optical, Tokyo, Japan). Ten random fields per plate were assessed, and the number of
keratinocytes containing melanosomes was counted. Pigment transfer is represented as a percentage of the control.

**Quantification of cell morphology and migration**

MNT1 melanoma cells were seeded at 10,000 cells/cm² and cultured to a confluency of 80% to minimize cell–cell contacts. Time-lapse experiments were performed on a microscope (Axiocert 200M, Zeiss, Jena, Germany) equipped with a microscope incubator to control temperature, humidity and CO₂. The positions of the cells were captured by tracking the nucleus of individual cells from a series of time-lapse images, taken at 5-min intervals for 6 h (approx. 250 cells per condition). Cell migration was manually tracked using Image J (NIH, Bethesda, MD, USA). During the analysis, cells were categorized into two groups according to their morphology. Cells that maintained their bipolar shape were classified as ‘bipolar cells’, while cells undergoing dynamic shape changes between rounded and multipolar were classified as ‘shape-changing cells’. For quantitative analysis, the average speed and root mean squared displacement (RMSD) of cell migration were analysed. The average speed indicates how fast the cells move and is calculated by dividing the total length of the migration path by the total observation time (6 h). RMSD indicates the spatial extent of migrating cells and was calculated from the ensemble average of the cell population, where n is the total number of cells from the time-lapse images.

**Statistical analysis**

All statistical analyses were performed with MINITAB® software (Minitab Inc., State College, PA, USA). To calculate a P value for comparisons between two samples, statistical analyses were performed using Student’s t-test. The threshold of significance was set at P < 0.05.

**Results**

**Dendrites are suppressed on softer substrates**

One distinctive morphological feature of melanocytes and melanoma cells is long arm-like protrusions called dendrites. These long dendrites become extended to transfer melanosomes more effectively to neighbouring keratinocytes upon stimulations such as UV irradiation. When NHMs and MNT1 cells were plated on a hard plastic dish or a glass whose elastic modulus is in the GPa range, almost all cells exhibit multiple long and slender dendritic processes even without UV irradiation. When these cells were cultured on PDMS substrates of decreasing stiffness, the lengths of the protrusions became significantly shorter, and the fraction of round cells increased significantly (Fig. 1a,b). For quantification, the morphologies of MNT1 cells were categorized into two distinct types, namely dendritic cells and round cells. We then counted the numbers of each morphological type to calculate the relative ratio between them for >2000 cells in two separate experiments. As shown in Fig. 1a,b, the number of round cells increased significantly as the stiffness of PDMS substrates decreased after 2 days of culture on laminin-coated PDMS for both cell types. Such stiffness-dependent morphological response became more pronounced at Day 4 for NHMs, whereas it seemed attenuated in MNT1 cells over time. The differential response between two cell types may attribute to the difference in cell densities. As shown in Fig. 1, normal NHMs have no sign of overgrowth at Day 4, whereas melanoma MNT1 cells of the same seeding density become overly populated at Day 4. Increase in cell–cell contacts at high confluency may counter effect on the stiffness-dependent responses of MNT1 cells by attenuating the response.

**Melanogenesis-related genes are suppressed on softer substrates**

The main function of melanocytes is melanin synthesis, termed melanogenesis. Tyrosinase, the key enzyme in melanogenesis, catalyses the hydroxylation of tyrosine to dihydroxyphenylalanine (DOPA) and its further oxidation to DOPA-quinone, which is thought to be the critical and rate-limiting step in melanin synthesis (16,17). MITF is a transcriptional regulator of tyrosinase gene expression and tyrosinase-related protein 2 (TRP2) is dopachrome tautomerase also involved in melanogenesis. We first used qRT-PCR and Western blotting to evaluate the profiles of gene transcription and protein expression for MITF, tyrosinase, TRP2, as well as a melanosomal structural protein, gp100/PME1L17 in NHMs cultured for 2 and 4 days on laminin-coated PDMS substrates of varying stiffness (Fig. 2a,b). While both gene and protein expressions of TRP2 (Fig. 2a,b) were decreased significantly on a softer stiffness, neither MITF nor tyrosinase exhibited any sensitivity to the differences in stiffness. We then tested the stiffness-dependent melanin synthesis of NHMs by measuring the melanin content at 490 nm as shown in Fig. 2c, and found that the cells’ ability to produce melanin indeed were influenced by the
that the cell–cell contacts upon growth of the cells may be playing a role in this observed desensitization. Alternatively, it is possible that the responsiveness of MNT1 cells to substrate stiffness is a transient behaviour in which MNT1 melanoma cells adapt to their microenvironment over a prolonged culture time, leading to apparent insensitivity to stiffness in 4 days. To test this hypothesis, we lowered the seeding densities to 5 \times 10^5 cells/sheet (by a factor of 4) and 1 \times 10^6 cells/sheet (by a factor of 20) and cultured until the cells of these two seeding densities reached the confluency observed in Day 2 sample of 2 \times 10^5 cells/sheet where no overgrowth was observed (Fig. 2f). In these conditions where cells were not very populated featuring minimal cell–cell contacts, the effects of the substrate stiffness became more apparent for a prolonged culture for 6 days. This result confirms that 1) MNT1 cells do respond to different stiffness by having different morphologies and levels of Tyrosinase production, and 2) cell–cell contacts (or overgrowth) may a counter-effect on the stiffness-dependent responses of MNT1 cells by attenuating the response, whose underlying mechanism is not known yet. Therefore, the effect of substrate stiffness on melanogenesis of MNT1 melanoma cells was not likely to be transient but must have been diluted in cell population of high density by increased cell–cell contacts.

**Melanin transfer from NHMs or MNT1 cells to NHks is suppressed on a softer substrate**

We tested the effects of different substrate stiffness on melanosome transfer from NHMs or MNT1 melanoma cells to NHks by co-culturing NHMs and NHks or MNT1 and NHks on PDMS substrates of different stiffness for 4 days. Because the effectiveness of melanosome transfer is known to be associated with dendricity, which is highly affected by substrate stiffness as shown in Fig. 1, we expected that melanosome transfer would also correlate well with the substrate stiffness. Our results indicate that melanosome transfer from NHMs or MNT1 human melanoma cells to neighbouring NHks was effectively increased on stiffer substrates, potentially assisted by a higher number of dendrites (Fig. 3a,b).

**Morphological stability of melanoma is reduced on softer substrates**

Recent findings have indicated that melanin transfer is achieved by the shedding of melanosome-rich packages, mostly from the dendritic tips, which are subsequently phagocytosed by the nearby keratinocytes (18) where they show the transfer is a rather slow process that takes nearly an hour. Therefore, for the effective transfer of melanin from melanocytes to nearby keratinocytes, it would be desirable to maintain stable dendrites during the transfer. Our time-lapse images of MNT-1 cells indicated that cells can be grouped into two categories based on their morphological stability. The first category is stable bipolar cells, and the second is unstable cells undergoing dynamic shape changes from rounded and multipolar to bipolar (Fig. 4a). A typical time scale for the change between dendritic and round shapes is on the order of 5–10 min, which is significantly faster than the time scale for melanin transfer (Fig. 4b). Thus, we may hypothesize that stable bipolar cells are the key players responsible for melanin transfer, whereas the shape-changing cells do not contribute much. When we compare MNT-1 cells grown on the stiffest (5.5 MPa) to those on the softest (0.05 MPa) PDMS substrates, we noticed a significantly high number of dynamic (or unstable) cells on the soft substrate compared to the stiff substrate (Fig. 4a). The presence of a
much larger fraction of stable bipolar cells on a stiff substrate is consistent with our hypothesis that cells on the stiff substrate would transfer melanin more efficiently.

We then characterized the migratory behaviour of MNT-1 cells by quantifying the average speed and the root mean squared displacement on PDMS substrates of two different stiffnesses. The stable bipolar cells exhibited slow translational motion along their long axis with oscillating nucleus back and forth; retraction of dendrites was rarely observed for the stable bipolar cells. By contrast, the shape-changing cells dynamically retracted and re-extended their dendrites while jumping around in all directions. Fast retraction of dendrites resulted in a relatively high displacement (thus high speed) of the cells. On both stiff (5.5 MPa) and soft (0.05 MPa) substrates, shape-changing cells exhibited higher average speed than bipolar cells, and for both morphological types, cells tended to move faster on the soft substrate of 0.05 MPa (Fig. 4d). Moreover, the transient round state of shape-changing cells permitted an unbiased choice of directions for cells to make a subsequent move, leading to more randomized motion with higher scatter, represented by a higher value in RMSD (Fig. 4e). Our motility assay demonstrated that cells of the two different shape categories migrated in a distinct manner; shape-changing cells migrated faster and further, exhibiting a frequent shifting of the centre of mass, while bipolar cells oscillated their nuclei without significant net displacement with stably maintained dendrites.

How do cells sense and respond to different stiffness? Cells of various types sense the substrate rigidity through focal adhesion complex via integrin receptors, and typically lower stiffness (at least within the range we are dealing with) has been correlated with weaker adhesion, which is considered as one of the landmark traits for stiffness-dependent cellular responses (19). Focal adhesion kinase (FAK) has been reported as an important component in signal transduction pathway transmitted from extracellular matrix (ECM) and integrin interaction (20). Because we observed dramatic changes in morphological stability in MNT1 cells on softer substrates, we performed western blot for p-FAK and total FAK of MNT1 cells to characterize the adhesion properties on the substrates of different stiffness (Fig. 4f). We observed that protein levels of both p-FAK and total FAK were decreased with the decrease in stiffness of PDMS substrate. Thus, FAK does seem to play a significant role in the stiffness-dependent motility response of MNT1 cells. Furthermore, because higher expression of integrin alpha 6 is known to

Figure 3. Decreased melanosome transfer between cells cultured on the soft substrate. Normal human melanocytes (NHMs) or MNT1 melanoma cells were co-cultured with NHKs for 4 days on laminin-coated polydimethylsiloxane of different stiffnesses. The cells were double-immunostained with anti-gp100/PMEL17 antibody (red) to detect melanosomes and anti-keratin antibody (green) to identify NHKs in the mixed cell culture (a). The inserts images show gp100/PMEL17-positive red spots in NHKs. For statistical analysis, the number of NHKs containing red spots in NHKs. For statistical analysis, the number of NHKs containing MNT1 cells were counted and reported as the percentage of total cell number (b) in 10 random microscopic fields. The values shown represent the mean ± SD of at least two independent measurements. *P < 0.05. Scale bars: 20 μm.

Figure 4. Morphological stability of melanoma is reduced on the soft substrate. MNT1 cells were cultured on laminin-coated polydimethylsiloxane of different stiffnesses. (a) MNT-1 cells were grouped into two categories, stable bipolar cells and unstable cells undergoing dynamic shape changes between rounded and multipolar to bipolar. The insert images show gp100/PMEL17-positive red spots in NHKs. For statistical analysis, the number of NHKs containing melanosomes was counted and reported as the percentage of total cell number (b) in 10 random microscopic fields. The values shown represent the mean ± SD of at least two independent measurements. *P < 0.05. Scale bars: 20 μm. (c) Time-lapse images of MNT-1 cells. A typical time-scale for the shape change between rounded and dendritic and round was on the order of 5–10 min. (d) Directionality of cell shape-dependent MNT1 cell movement on substrates of different stiffnesses. Cells on stiffer substrates have higher directionality than those on softer substrates, and cells with bipolar shape have higher directionality than shape-changing cells on both stiff and soft substrates. (d) Average migration speed of MNT1 cells on different stiffness. MNT1 cells on the stiff substrate migrated significantly slower than cells on the soft substrate. The data represent means ± SE (error bars) of more than 60 cells pooled from two independent experiments. *P < 0.05 and **P < 0.01. The effects of the substrate stiffness on the expression of phospho-FAK, total FAK and tyrosinase in MNT1 cells seeded at low density of 5 × 10^4 for 4 days and 1 × 10^5 for 6 days of growth were determined by Western blotting analysis (f).
correlate positively with attachment to laminin and the dendrite formation of MNT1 cells may be mediated by integrin alpha 6.

**Discussion**

In this study, we showed the substrate stiffness-dependent behaviour of NHMs and MNT1 melanoma cells in the context of pigmentation. While not all responses were same between NHMs and MNT1 cells, the stiffness-dependent morphological changes and the ability to transfer melanin to NHKs were consistent in both cell types, and at least one of the melanogenesis-related genes/proteins tested showed stiffness-dependent alterations in both cell types. These results confirmed the generality of our conclusions that the stiffer substrates induce higher dendrite formations, higher melanin transfer, and melanogenic gene and protein regulations in melanocytes and melanoma cells.

Integrin and FAK are known to influence migration of melanocytes over extracellular matrix (22). The consistent decrease in expression of integrin alpha 6 and FAK in MNT1 cells cultured on a softer substrate implies weakening of adhesion strength, which correlates well with cells’ dynamic shape changes that feature frequent jumping, resulting in randomized scattering with higher RMSD on a softer substrate. Because there was a lower fraction of round cells on a stiffer substrate, the average movement of MNT1 melanoma cells cultured on stiff substrates (5.5 MPa) was lower on average, and the cells tended to aggregate locally, with a small scatter. The phenotypes of MNT-1 cells, both morphological and migratory, were more stable on a stiff substrate, which may explain the effectiveness of melanin transfer on a stiff surface.

When adherent cells are not properly spread either by culturing on a soft matrix or by forcing them into the geometric constraint of a small area, cells can lose their normal functionality and undergo apoptosis (23). Therefore, one possible explanation for the observed suppression of dendrite outgrowth and melanogenesis on a soft substrate of 0.05 MPa could be that cells did not bind strongly enough to the soft substrate, preventing the cells from extending their dendrites and regulating their normal pigmentation function. To confirm that MNT1 cells on a soft substrate are rounded but otherwise functional and capable of extending dendrites, we treated MNT1 cells with 50 µM forskolin for 2 days. Forskolin is a labdane diterpene that activates the enzyme adenyl cyclase and increases the intra-cellular levels of cyclic AMP, similar to the induction of skin pigmentation by UV radiation through the upregulation of melanogenic genes and melanocyte dendrities. As shown in Figure S2a, MNT1 melanoma cells cultured with forskolin on the softest PDMS substrate (0.05 MPa) exhibited long dendrites that were morphologically similar to those grown on the stiffest substrate (5.5 MPa) and the total cell number of MNT1 cells cultured with or without forskolin for 2 days was not changed by PDMS substrate stiffness (Figure S2b). The indistinguishable morphologies in forskolin-treated MNT1 cells across different stiffness correlate well with flat gene expression levels shown below (Figure S2), implying that MNT1 melanoma cells on the softest PDMS substrate responded to the pigmentary stimuli, inducing melanogenic gene expression and dendrite outgrowth. The softness of the substrate has an effect on the basal melanogenic ability and the morphology of the pigment cells but does not inhibit the responsiveness of cells to a physiological stimulator.

From these results, we suggest that skin pigmentation is influenced by the mechanical properties of the cellular microenvironment through adhesion dependent pathway and it increases when the skin becomes stiff. Human skin hyperpigmentation disorders, such as melasma, postinflammatory melanoderma and solar lentigo, are aesthetically undesirable conditions. In case of skin fibrosis with excess fibrous, connective tissue, which is known to be stiffer than surrounding healthy skin, is often accompanied by skin pigmentation (6,7). Moreover, as previously mentioned, skin hyperpigmentation regions such as melamomas and junctional nevi (JN) exhibit greater stiffness than normal surrounding skin (5). Therefore, we believe that our finding on the stiffness-dependent behaviour of melanoma and normal melanocytes would be able to give new insights into the factors that regulate skin pigmentation and provide foundations for the development of an agent to treat undesirable skin hyperpigmentation by reducing the mechanical stiffness around melanin-producing cells in the skin.

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**Author contribution**

HC performed qRT-PCR, Western blotting and melanosome transfer experiments. MK and SA prepared PDMS substrates and performed motility experiments. HC, EC, TL and JS designed the research and drafted the storyline. HC and JS wrote and revised the paper.

**Conflict of interests**

The authors have declared no conflicting interests.

**References**


**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Quantification of laminin coating on substrates of different stiffness.

**Figure S2.** Effects of forskolin on cellular morphology and the mRNA expression of melanogenesis-associated and adhesion genes in MNT1 melanoma cells cultured on substrates of different stiffnesses.