RSC Advances

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/advances

Long term effects of substrate stiffness on the development of hMSC mechanical properties

Qinwei Xu[1]†, Cheng Li[1, 2]†, Kang Yuejun[3], Yilei Zhang[1*]

1. School of Mechanical and Aerospace Engineering, Nanyang Technological University, Singapore
2. Singapore Centre for Environmental Life Sciences Engineering, Interdisciplinary Graduate School, Nanyang Technological University, Singapore
3. School of Chemical and Biological Engineering, Nanyang Technological University, Singapore

†. They contributed equally to this work

*. ylzhang@ntu.edu.sg

ABSTRACT

Human mesenchymal stem cells (hMSCs) are multipotent stem cells with promising potential in tissue engineering and cell therapy applications. The mechanical properties of hMSCs are associated with their behaviors, like adhesion and differentiation, and can be influenced by mechanical properties of substrates that they adhere to. The objective of this work is to investigate the effect of substrate stiffness and culture time on the elastic and viscoelastic properties of hMSCs. Particularly, polydimethylsiloxane (PDMS) substrates with different stiffness were prepared for long-term hMSC culture. Micropipette aspiration method was applied to measure the mechanical properties of hMSCs at different time points during the culture period. Elastic and viscoelastic properties of hMSCs were evaluated using two-way analyses of variance (ANOVA). Results showed that cells aligned their mechanical properties according to the substrate stiffness and became softer on softer substrates and stiffer on stiffer substrates. Furthermore, regardless of PDMS substrate stiffness, cell moduli as functions of time displayed an non-monotonic trend, which could be classified into three different zones: 1) adapting zone, where cellular moduli decrease at the very beginning to adapt to the new substrates after taking out from a flask and the adapting time required is proportional to the difference of the two substrates in stiffness; 2) growth zone, where cellular moduli increased to the maximum values; and 3) convergent zone, where cellular moduli decrease again to similar values regardless of substrate stiffness.

KEYWORDS

Micropipette aspiration, polydimethylsiloxane, viscoelasticity, cellular mechanical properties, hMSCs

INTRODUCTION

Mesenchymal Stem Cells (MSCs) are multipotent stem cells which can be isolated mainly from bone marrow. Other tissues such as liver, amniotic fluid, and placenta could also be their sources (1). The significance of studying MSCs lies in their ability to differentiate into different cell lineages (2) as well as their promising potential to be applied to cell and gene therapy for serious diseases like heart failure or hematological pathologies (1).

Cellular behaviors of MSCs including locomotion, proliferation, and differentiation depend a lot on their growing microenvironment (3–6). Because it is necessary for tissue cells to adhere to and grow on solid surfaces (7), the influence of substrate properties, like stiffness, on cells has been extensively studied (4, 8–12). It has been found that the human Mesenchymal Stem Cells (hMSCs) became stiffer when cultured on stiffer substrates (4). With or without induction factors, it has been suggested that the stiffness of substrates could direct the lineage specification of hMSCs (9, 13) and the viscoelasticity of hMSCs changed monotonically with time during differentiation (14). It was also shown that MSCs grew on soft substrates tended to be neurogenic while on stiffer substrate tended to be myogenic or even osteogenic (9). The culture time on substrates with different stiffness could even influence the following differentiation behavior of the MSCs. MSCs could easily differentiate into different lineages when induction factor was added after a short time period of culture on the substrates while they became difficult to be induced to other lineages except the substrates-defined lineage after a long time period of culture on

the substrates (9). For example, if the MSCs were cultured on the soft neurogenic matrices for three weeks, they could only be induced to neurons even if myogenic or osteogenic factors were added into the media. Nonetheless, a good method to distinguish the potential of stem cells to differentiate into specific lineages was yet to be established (15). Recent studies confirmed the feasibility of correlating differentiation behavior of stem cells with their mechanical properties, for example, osteogenesis was positively correlated with the elastic as well as viscous properties of the cells (14, 15). Therefore, cell mechanical property measurement could serve as an easy and effective characterization of cellular behaviors.

Micropipette aspiration (MA) has been widely applied to measure cell mechanical properties (14, 16–21), which is based on the detection of the cellular deformation under a given external pressure. Cells are connected to the external environment through cellular cytoskeleton. From either extracellular matrix or neighbors, cells could sense the external signals that guide complex behaviors such as migration and differentiation. The cell deformation is driven by the polymerization and de-polymerization of actin filaments and microtubules together with molecular motors. The internal or external mechanical forces could affect several classes of regulatory proteins involved in the cellular architecture, thus, the local organization of the cytoskeletal network (22). For example, recent studies have demonstrated that the contractile force of cellular actin-myosin cytoskeleton, which was determined mainly by cells' sensing of the surrounding extracellular matrix, could affect cytoskeletal organization (7). Compared with other commonly used methods, such as atomic force

microscopy (AFM) (23, 24) and optical traps (25, 26), MA method has the ability to generate large forces and measure the global mechanical properties of the investigated cell. Depending on the cellular behavior in the micropipettes, various models have been developed to extract cell mechanical modulus (17, 27). For hMSCs, both elastic and viscoelastic solid models have been used in previous studies (14, 20, 21).

However, the long-term relationship between substrate stiffness and mechanical properties of cells has not been well studied. The objective of this study was to investigate the long-term influence of substrate stiffness on the mechanical properties of hMSCs. Instead of the commonly used substrate, such as polyacrylamide (8, 9), polydimethylsiloxane (PDMS) substrate was adopted for the easy preparation procedure and easy stiffness control, which only requires modification of the ratio between the base material and the curing reagent. The hMSCs were cultured on PDMS substrate without adding any induction factor for a total duration of two weeks and measured at six time points – day 0, 1, 5, 7, 11, and 14. Three replicates were obtained for each sample and the data were analyzed using two-way analyses of variance (ANOVA) so that not only the influence of substrate stiffness but also the culture time could be evaluated.

MATERIALS AND METHODS

Fabrication of the PDMS Substrates with Different Stiffness

PDMS (Sylgard 184, Dow Corning, USA) substrates with different stiffness were prepared by mixing the base and the curing agent in varying weight ratios. Higher

ratios result into lower moduli due to lower crosslink densities (28). Same amount of PDMS mixtures with base to reagent ratios of 5:1 (PDMS5), 10:1 (PDMS10), 20:1 (PDMS20), and 30:1 (PDMS30) were poured into 24-well plates and a 1 mm thick substrate was formed in each well. The plates were then kept in a 60°C oven for 2h for PDMS solidification after degassing. As cell attachment is limited by the hydrophobic nature of PDMS (29, 30), poly-L-lysine (PLL, Sigma, Singapore) coating was conducted onto all substrates before cell seeding by immersing the substrates in PLL solution (0.01%) for 30 minutes. Even though some previous studies suggested that poly-L-lysine could facilitate the chondrogenic differentiation of hMSCs when specific induction factors were added into the cell growth media (31), no evidence showed that it could influence cell mechanics or induce cell differentiation directly. Thus, the PLL coating is mainly to promote cell adhesion(32–35). The moduli of different PDMS materials were characterized using an Instron machine.

hMSC Culture

Human Mesenchymal Stem Cell (hMSC) line derived from human bone marrow was purchased from Life Technologies, Singapore. After detaching from the culture flask using 0.25% Trypsin (Life Technologies, Singapore) for less than 4 mins, hMSCs were seeded onto the prepared PDMS substrates with a density of around 5,000 cells per well. Inoculated cells were kept in Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Singapore) supplemented with fetal bovine serum (FBS) (Life Technologies, Singapore.) and penicillin/streptomycin (Life

Technologies, Singapore), which was changed twice a week, before micropipette aspiration. Prior to micropipette aspiration, cells were detached using 0.25% Trypsin (Life Technologies, Singapore) treatment for less than 4 mins.

Fluorescent staining and imaging

Cells were fixed in the 10% neutral buffered formalin solution (Sigma, Singapore) for 20 min and permeabilized with 0.1% Triton X-100 (Sigma, Singapore) for 5 min followed by two times PBS wash. F-actin filaments were stained by 1 hour incubation with rhodamine-conjugated phalloidin (Life Technologies, Singapore) at 1:500 dilution at room temperature. To stain nuclei, each well was incubated with DAPI (Life Technologies, Singapore) at 1:1000 dilution for 5 min. Images were taken with an inverted fluorescent microscope.

Micropipette Aspiration

The micropipette aspiration system is illustrated in Fig. 1. A micromanipulater was used to move a micropipette with a sharp tip close to a cell. A microinjector was used to generate the pressure applied to the cell, which would be measured using a pressure transducer, a demodulator, and a digital multimeter. A microscope and a computer were used to locate the cells and record the images during the aspiration. Micropipettes of inner diameters from 8μm to 12μm, which are around 0.2 to 0.8 times of cell diameters, were obtained by drawing borosilicate glass capillary tubes (1.0 mm outer diameter, 0.5 mm inner diameter, Sutter Instrument, USA) with a horizontal pipette puller (Sutter Instrument, USA). At the beginning of an experiment,

the whole system was filled with deionized (DI) water using the syringe to remove air bubbles trapped in the system. After that, the cell of interest was placed in the middle of the view field under the microscope by moving the microscope stage. Then the micropipette was moved close to the cell using the micromanipulater and an equilibrium pressure, under which the cell was neither sucked in nor pushed away, was achieved through the microinjector (Narishige, Japan) before an aspiration pressure was applied to deform the cell. The applied pressure was measured by a pressure transducer (Validyne Engineering, USA), converted to voltage values by a demodulator (Validyne Engineering, USA), and displayed with a digital multimeter (Fluke, USA). A total of 30 images were taken during the around 300s aspiration process using an inverted microscope (Nikon, Japan) attached with a camera (Tucsen, China). Open source software (ImageJ by NIH, USA) was used for the image processing to get the aspiration length data.

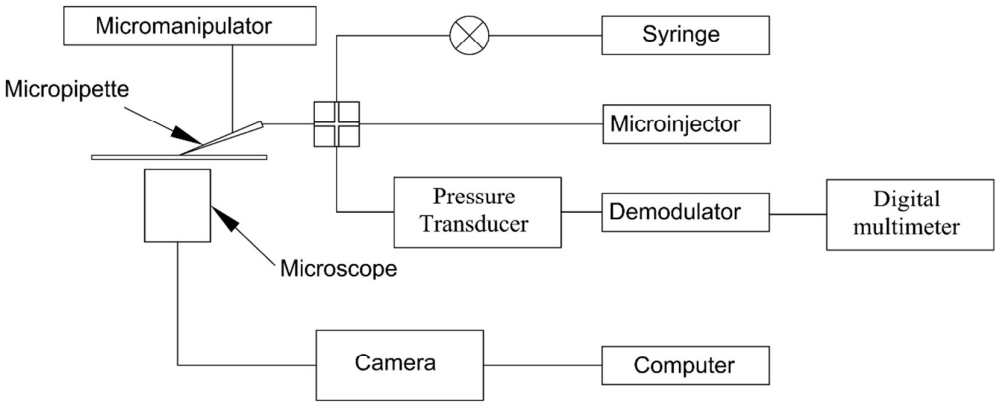

Figure 1 Micropipette aspiration system illustration.

Theoretical Modelling

A linear elastic model and a viscoelastic model, in which the cell is assumed to be homogeneous (27), were used to extract the mechanical properties of hMSCs. The linear elastic model is a simplification of viscoelastic model through ignoring the time factor. In this model, cell is approximated as an incompressible elastic half-space (17, 27, 36), i.e., the local radius of the cell surface is relatively large compared to the pipette radius R_p. The elastic modulus E of the cell is calculated as (36):

$$E = \Phi \left(\frac{3R_p}{2\pi}\right)\left(\frac{\Delta P}{L_a}\right) \quad \text{Eq. 1}$$

where L_a is the equilibrium aspiration length, ΔP is the applied aspiration pressure, and Φ is a constant related to the ratio of the pipette wall thickness to the pipette radius, which is chosen to be 2.0 in this study based on the *punch* model (36).

In the viscoelastic model, three parameters (Fig. 2) were obtained through evaluating the real time influence of applied aspiration pressure on the aspiration length ($L(t)$) of the cell. The relationship between $L(t)$ and the time passed since the pressure is applied could be fitted using the following equation (37):

$$L(t) = \frac{\Phi R_p \Delta P}{\pi k_1}\left[1 - \frac{k_2}{k_1+k_2}e^{-t/\tau}\right] \quad \text{Eq. 2}$$

where Φ, R_p, and ΔP have same definition as in the elastic model (Eq. 1). The apparent viscosity μ is given by (37):

$$\mu = \frac{\tau k_1 k_2}{(k_1+k_2)} \quad \text{Eq. 3}$$

where τ is the exponential time constant while k_1 and k_2 are the elastic constants. Both constants are needed to calculate the standard elasticity coefficients:

$$E_0 = \frac{1}{2}(k_1 + k_2) \quad \text{Eq. 4}$$

$$E_{eqm} = \frac{1}{2}k_1 \quad \text{Eq. 5}$$

where E_0 is the instantaneous Young's modulus determined before the aspiration and E_{eqm} is the equilibrium Young's modulus determined at the point that suction length was no longer increased (20, 21).

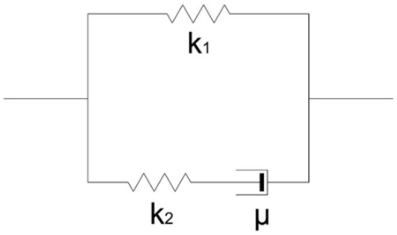

Figure 2 Illustration of the viscoelastic model, where k_1 and k_2 are the elastic constants of two springs; μ is the viscous constant of a dashpot.

Statistical Analysis

The elastic data of PDMS substrates were analysed using one-way analyses of variance (ANOVA) while two-way ANOVA method was applied for the elastic and viscoelastic data of cells, with PDMS stiffness and culture time as two independent variables. Tukey's post-hoc tests were performed when significance was indicated in ANOVA results. Significance was determined at $p<0.05$ and a trend towards significance was determined at $p<0.1$. All values were reported as mean ± standard error of the mean (SEM).

RESULTS

Stiffness of Different PDMS substrates

The Young's moduli of different PDMS measured using Instron extension test showed a clear trend that with higher base to curing agent ratios the PDMS became softer (Fig. 3). And the Young's moduli of PDMS substrates fall into the range from 0.06 MPa to 1.2 MPa, which could cover the stiffness range from muscle to bone (38).

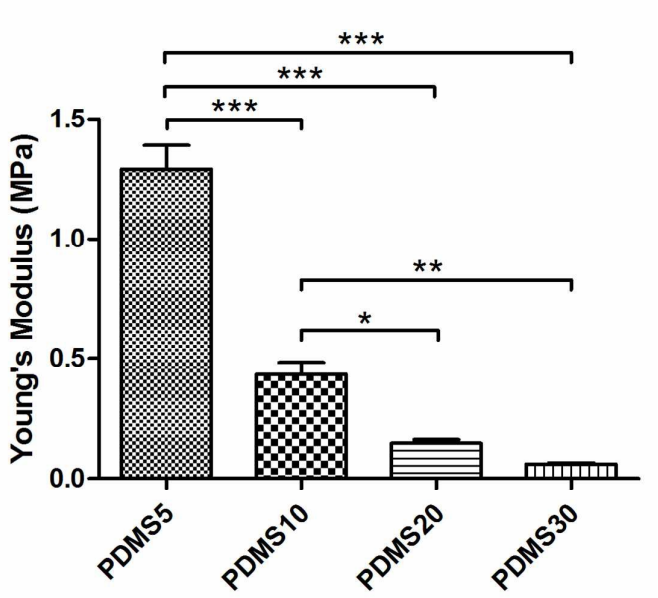

Figure 3 Young's modulus of PDMS measured by Instron machine (* indicates significant difference between the two groups with $p<0.05$, ** for $p<0.01$, and *** for $p<0.001$)

Morphology of hMSCs on PDMS

Optical images of hMSCs on different PDMS were monitored throughout the experiment period (Fig. 4). It could be observed that under current seeding density (5,000 cells per well), hMSCs were growing individually without clustering. Compared the initial growth stages (Fig. 4(A) and 4(D)) with the later stages (Fig. 4(C)

and 4(F)), cells were separated away from each other on both PDMS5 and PDMS30 without clustering or forming a closely packed monolayer. Therefore, the measured cellular modulus should mainly be affected by the PDMS stiffness.

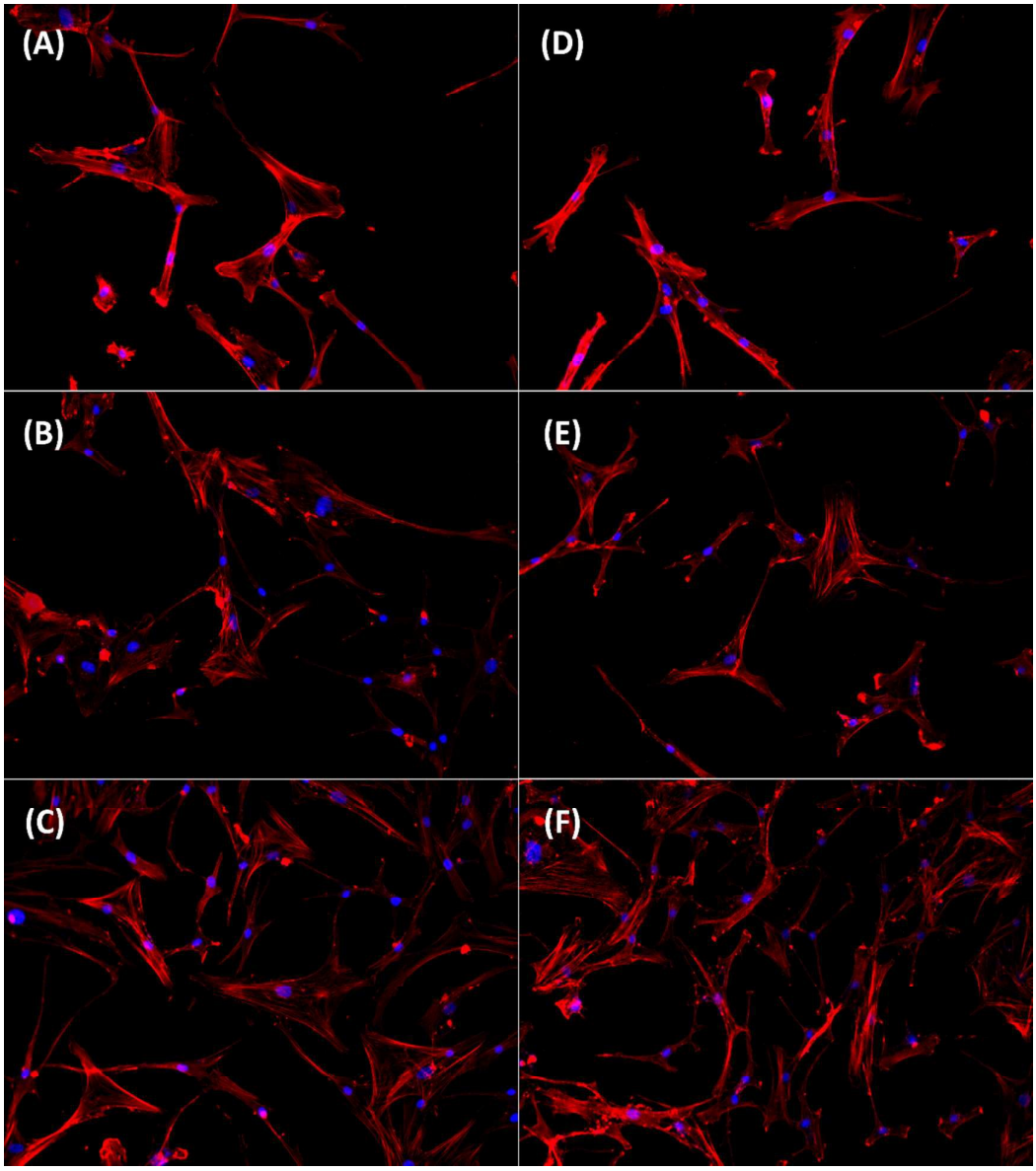

Figure 4 Fluorescent images of hMSC cultured on PDMS. Left: Day 5 (a), Day 11 (b) and Day 14 (c) on PDMS5; Right: Day 5 (d), Day 11 (e) and Day 14 (f) on PDMS30. Red: Rhodamine Phalloidin stained F-actin; Blue: DAPI stained nuclei

Elastic Property of hMSCs

To evaluate the elastic property of hMSCs, elastic moduli of cells were calculated from the elastic model. In the situation when pipette radius is much smaller than cell radius (27), the cell could be approximated as an incompressible elastic half-space (36) and the simplest elastic model could be used to describe the cell aspiration behavior.

To examine the effects of PDMS stiffness and culture time on cell elastic moduli, the results derived from the elastic model were displayed (Fig. 5). Results of the two-way ANOVA test gave the main effect value for PDMS stiffness, $F(3, 40)=4.248$, $p=0.0107$ and the main effect value for culture time, $F(4,40)=16.41$, $p<0.0001$, which indicated that both PDMS stiffness and culture time had significant influence on the elastic moduli of cells. However, the interaction between PDMS stiffness and culture time, with main effect value of $F(12,40) = 0.5742$, $p=0.8495$, had no significant effects.

Elastic modulus of the hMSCs detached from the normal culture flask (Day 0) was the highest (Fig. 5A), which was around 516 Pa. On day 1, the elastic modulus of cells cultured on all PDMS substrates had similar values to each other around 100 Pa, which decreased significantly from Day 0. This could be attributed to the large stiffness change from the culture flask (~1 GPa) (39) to the PDMS substrates. On day 5, Young's moduli of cells were 155±76 Pa on PDMS5, 102±16 Pa on PDMS10, 41±5 Pa on PDMS20, and 35±6 Pa on PDMS30, which shows a clear trend that the cells cultured on stiffer substrates had higher moduli. On day 7, similar trend could be observed except that the Young's modulus for PDMS20 was larger than that for

PDMS10. On day 11, a trend towards significant difference of cell moduli between PDMS5 and PDMS30 was shown. Finally on day 14, moduli of cells on different PDMS substrates became similar again, which were around 180 Pa. In general, the moduli of the cells cultured on stiffer substrates were larger than the cells grown on softer substrates from day 5 to 11, with exceptions found in only two groups, PDMS10 & PDMS20 on day 7 and PDMS5 & PDMS10 on day 11, among the 18 comparison pairs.

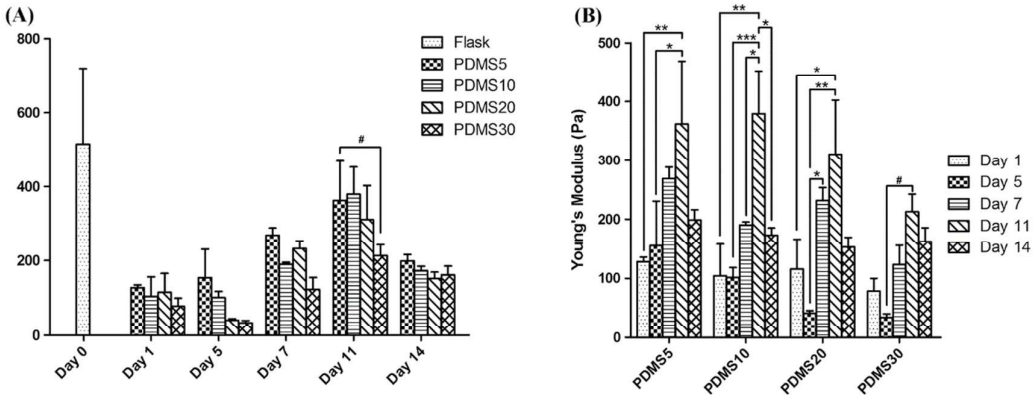

Figure 5 Young's modulus of cells calculated using elastic model ((A) modulus changing with time, (B) modulus changing with PDMS stiffness) (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and # means $p<0.1$)

Besides the influence of substrate stiffness, the culture time played an even more significant role on the development of cellular moduli (Fig. 5B). Overall, cellular moduli showed an increasing and then decreasing trend from day 5 to day 14. The largest moduli were always achieved on day 11 and the smallest moduli on day 5 for all PDMS substrates. As an example, for cells grew on PDMS5, which was the stiffest substrate, cell moduli increased from 155±76 Pa on day 5 to the maximum 362±107

Pa on day 11. After that, the value decreased to 199±17 Pa on day 14. Cell moduli on day 1 were larger than on day 5 except PDMS5, which might indicate that how fast the cells could adapt to the substrate stiffness depended on how large the substrate stiffness was changed. In details, the experimental results indicate that it took more than 1 day for hMSC to adapt to the big change in substrate stiffness when transferred from the stiff flask to soft PDMS substrates (PDMS 10, 20 and 30). As a results, cells did not fully adapt to the soft substrate on Day 1 and would continue reduce the after that, which made cellular moduli larger on Day 1 than Day 5. While for PDMS5, which was more than 2 times stiffer than other PDMS substrates, it took less than 1 day for hMSC to adapt to the substrate stiffness change so that cellular moduli could show a monotonic increase.

Viscoelastic properties of hMSCs

hMSCs in the experiments showed typical viscoelastic behavior during aspiration. Right after the step pressure being applied, there was an aspiration length jump. Following the initial jump, the aspiration length increased slowly under constant pressure before reached the equilibrium length (Fig. 6). Through fitting the time dependent aspiration length using the viscoelastic model, three relevant mechanical parameters, instantaneous Young's modulus (E_0), equilibrium Young's modulus (E_{eqm}), and apparent viscosity (μ), could be obtained (Fig. 7, 8). All the aspiration length data were fitted using the viscoelastic model as explained in the methods section (Equ. 2). Most fittings showed high accuracy with the mean correlation coefficients higher than 0.9. The worst fitting had a mean correlation coefficient

around 0.71, which was probably due to the broke of the cell membrane during aspiration process. Overall, results showed that the instantaneous Young's modulus of cells (in the range of hundreds or even thousands of Pascal) were larger than their equilibrium Young's modulus (in the ranger of tens to hundreds of Pascal). And most of the apparent viscosity values were in the range of thousands of Pascal*second.

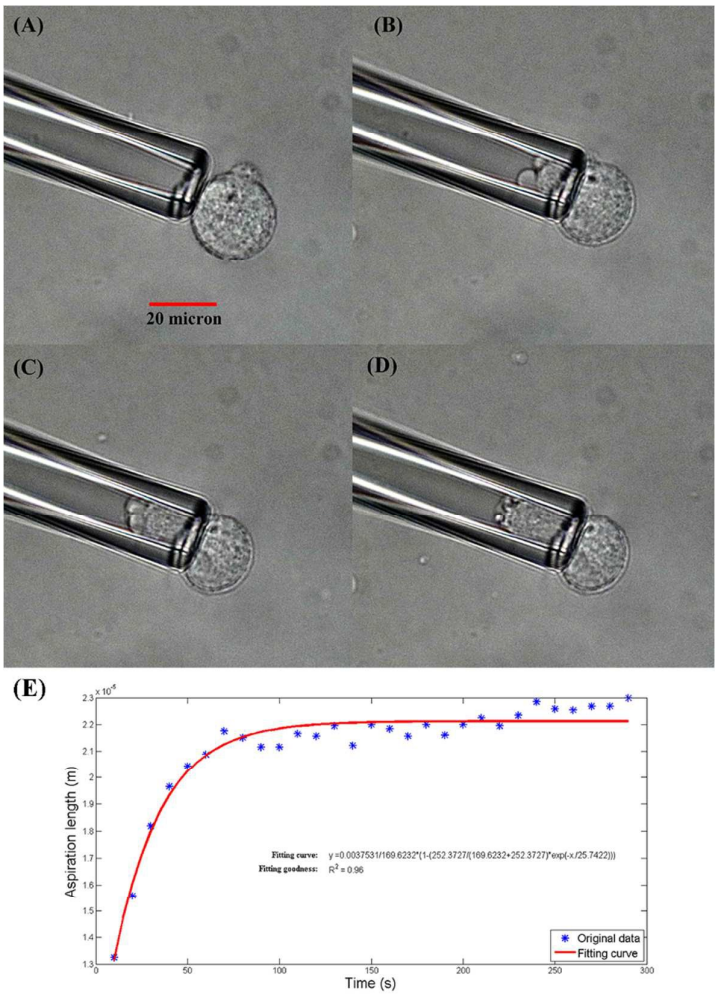

Figure 6 Aspiration of cells in micropipette at different time points. (A) the pressure was adjusted to reach equilibrium, (B), (C), and (D) were 0s, 100s, and 300s after applying the step aspiration pressure, and (E) was the corresponding fitting curve of the cell aspiration

length as a function of time

The main effects of PDMS stiffness and culture time on the three viscoelastic parameters, E_0, E_{eqm}, and μ were evaluated as well using two-way ANOVA method. Main effects of PDMS stiffness ($F(3,40)=6.16$, $p=0.0015$) and culture time ($F(4,40)=11.30$, $p<0.0001$) on E_0 suggested that both of them had significant influence. Similarly, main effects of PDMS stiffness ($F(3,40)=3.767$, $p=0.0180$) and culture time ($F(4,40)=18.38$, $p<0.0001$) on the E_{eqm} are also significant. However, the interaction between PDMS stiffness and culture time showed no significant effect on E_0 and E_{eqm}. Furthermore, effects of PDMS stiffness, culture time, and their interaction on μ were all insignificant.

A strong dependence of the cell mechanical properties on PDMS substrate stiffness could be noticed, which is the same trend as the elastic model but more obvious (Fig. 7). In general, the viscoelastic parameters of cells cultured on a stiffer PDMS were usually larger than those cultured on a softer PDMS from day 5 to 11 and the differences were larger than the elastic modeling results. Only 5 exceptions where cells had larger moduli or viscosity on softer PDMS were observed among the 54 one-to-one comparisons of all three viscoelastic parameters from day 5 to 11 between cells cultured on different PDMS substrates.

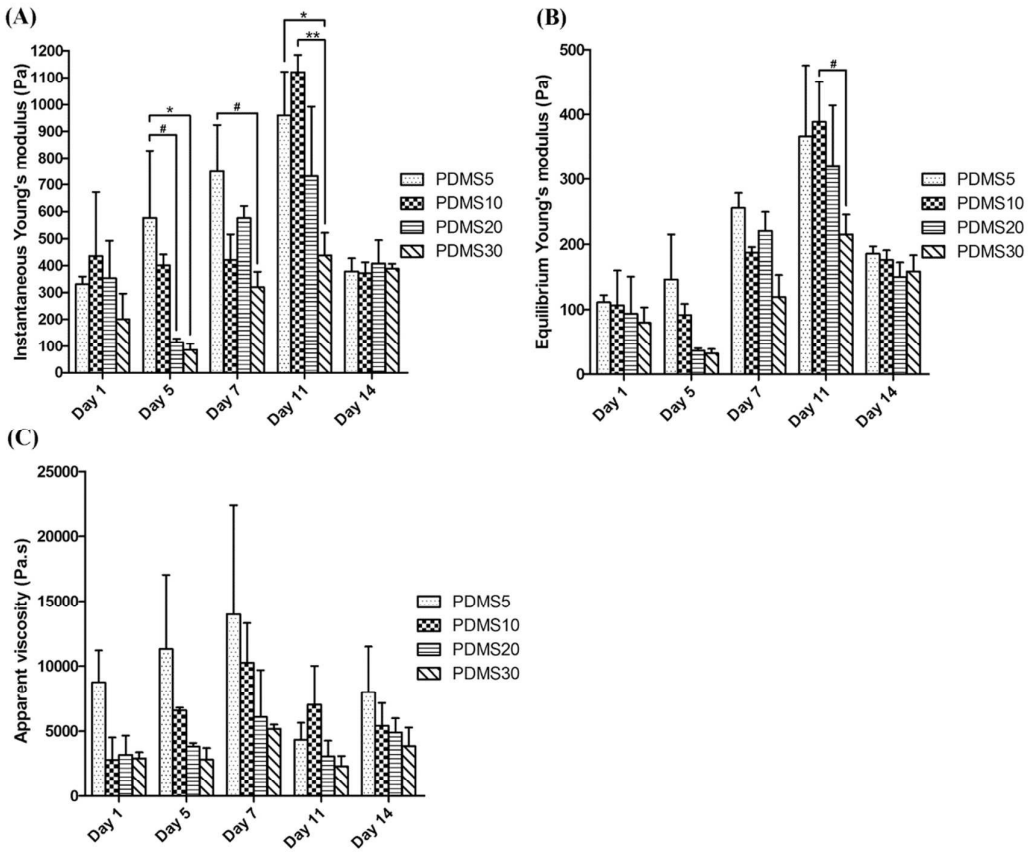

Figure 7 Cell viscoelastic parameters influenced by PDMS substrate stiffness ((A), (B), and (C) were figures for instantaneous Young's modulus, equilibrium Young's modulus, and apparent viscosity respectively as functions of culture days) (* indicates $p<0.05$, ** indicates $p<0.01$, *** indicates $p<0.001$ and # means $p<0.1$)

The influence of cell culture time on the three mechanical parameters was also studied (Fig. 8). In the two weeks experimental duration, the change of the instantaneous and equilibrium moduli with time was similar as the elastic model results. The instantaneous Young's moduli of cells cultured on PDMS5 increased first from day 1 to day 11 and then decreased on day 14, while instantaneous Young's moduli of cells cultured on other PDMS substrates decreased first from day 1 to day 5,

then increased from day 5 to day 11, and finally decreased again at day 14. Similarly, the difference of PDMS5 on day 1 may be attributed to the shorter time required for cells to adapt to the new substrate with smaller stiffness change. Exactly same phenomenon was shown for the equilibrium Young's modulus. For both E_0 and E_{eqm}, similar values were reached on Day 14 regardless of substrate stiffness. Different from the trends of E_0 and E_{eqm}, there is no any significant difference for cell apparent viscosity values at different time points.

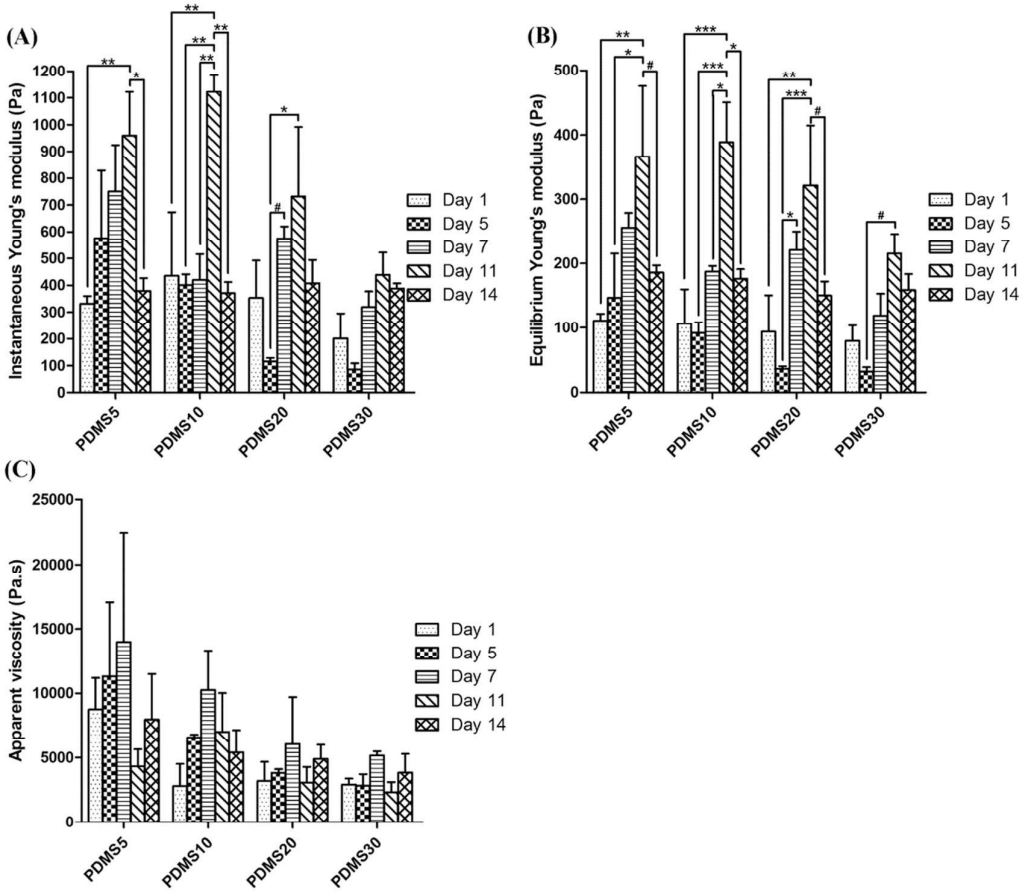

Figure 8 Cell viscoelastic parameters as influenced culture time ((A), (B), and (C) were figures for instantaneous Young's modulus, equilibrium Young's modulus, and apparent viscosity respectively as functions of PDMS stiffness) (* indicates p<0.05, ** indicates

p<0.01, *** indicates p<0.001 and # means p<0.1)

DISCUSSIONS AND CONCLUSIONS

Previous studies have demonstrated that cells could respond to the physical properties, including stiffness and topography, of the substrates (4, 9, 40, 41). It was suggested that the differentiation behavior of MSCs could be related to the substrate stiffness (9). On the softest substrates, MSCs showed the greatest expression of neurogenic transcripts by upregulating neuron-specific cytoskeletal markers such as nestin, β_3 tubulin, neurofilament light chain (NFL), and protein NCAM. On moderately stiff substrates, MSCs express the most myogenic message with upregulation of proteins such as Pax activators and myogenic factors (e.g. MyoD). On the stiffest substrates, MSCs express the greatest osteogenic message with upregulation osteocalcin and transcriptional factor CBFα1. The lowest substrate stiffness in this study was 60.75±8.62 kPa, which is already in the range that could direct MSCs to differentiate into osteogenic lineage.

Change of cell mechanical properties, such as elasticity and viscoelasticity, have attracted lots of interests due to its relationship with various cellular behaviors, like proliferation, adhesion and differentiation (14). According to the results, a strong dependence of the cell mechanical moduli on substrate stiffness could be observed, regardless of which model was used. In general, cell mechanical moduli tended to be larger on stiffer substrates, similar to previous works (4, 40). This tendency of cells tuning their stiffness to match that of the substrates they attached could be explained

by increased cytoskeletal assembly, activation of crosslinkers, and generation of internal tension which is related to the strain-stiffening non-linear elasticity of the cytoskeleton (40).

However, the influence of culture time should also be very important. Previous studies have shown an increasing stiffness of individual human epithelial cells with aging in vitro (42), which was attributed to a higher density of cytoskeletal fibers in the older cells than those in the younger cells that led to the increase of cellular modulus. This work also highlighted the effect of culture time on cellular mechanical properties. Particularly, it was found that cellular moduli as functions of time displayed a non-monotonic trend regardless of PDMS substrate stiffness, which could be classified into three different zones: 1) adapting zone, where cellular moduli decrease at the very beginning to adapt to the new substrates after taking out from a flask and the adapting time required is proportional to the difference of the two substrates in stiffness; 2) growth zone, where cellular moduli increased to the maximum values; and 3) convergent zone, where cellular moduli decrease again to similar values regardless of substrate stiffness.

This change of cell moduli observed through long-term culture indicated that cell stiffness should be modulated not only by substrate stiffness but also by culture time. According to the *p* values of main effects of PDMS and culture time, the impact of the culture time on the development of mechanical properties of hMSCs was greater than that of PDMS stiffness, i.e., culture time could have even more significant effects on cellular moduli than substrate stiffness.

Author contributions

QX and CL performed the experiments. YK contributed in the equipment. YZ designed the study. QX, CL and YZ wrote the paper. All authors have approved the final version of the manuscript.

Acknowledgement

We thank Shi-Ying Chan, Swee-Tong Chong and Ghim-Hian Goh for help during the experiment. Y.Z. acknowledges the Tier-1 Academic Research Funds from the Singapore Ministry of Education (RGT 30/13and RGC 6/13).

REFERENCE

1. Giordano, A., and U. Galderisi. 2007. From the laboratory bench to the patient ' s bedside : An update on clinical trials with mesenchymal stem cells. J. Cell. Physiol. 2 I I: 27–35.

2. Pittenger, M.F., A.M. Mackay, S.C. Beck, R.K. Jaiswal, R. Douglas, J.D. Mosca, M.A. Moorman, D.W. Simonetti, S. Craig, and D.R. Marshak. 1999. Multilineage potential of adult human mesenchymal stem cells. Science. 284: 143–147.

3. Bao, G., and S. Suresh. 2003. Cell and molecular mechanics of biological materials. Nat. Mater. 2: 715–725.

4. Tee, S.-Y., J. Fu, C.S. Chen, and P. a. Janmey. 2011. Cell shape and substrate rigidity both regulate cell stiffness. Biophys. J. 100: L25–L27.

5. Park, J.S., J.S.F. Chu, C. Cheng, F. Chen, D. Chen, and S. Li. 2004. Differential effects of equiaxial and uniaxial strain on mesenchymal stem cells. Biotechnol. Bioeng. 88: 359–368.

6. Park, S., W. Sim, and S. Park. 2006. An electromagnetic compressive force by

cell exciter stimulates chondrogenic differentiation of bone marrow-derived mesenchymal stem cells. Tissue Eng. 12.

7. Discher, D.E., P. Janmey, and Y.-L. Wang. 2005. Tissue cells feel and respond to the stiffness of their substrate. Science. 310: 1139–1143.

8. Lee, J., A.A. Abdeen, and K.A. Kilian. 2014. Rewiring mesenchymal stem cell lineage specification by switching the biophysical microenvironment. Sci. Rep. 4: 5188.

9. Engler, A.J., S. Sen, H.L. Sweeney, and D.E. Discher. 2006. Matrix Elasticity Directs Stem Cell Lineage Specification. Cell. 126: 677–689.

10. Kobayashi, N., T. Yasu, H. Ueba, M. Sata, S. Hashimoto, M. Kuroki, M. Saito, and M. Kawakami. 2004. Mechanical stress promotes the expression of smooth muscle-like properties in marrow stromal cells. Exp. Hematol. 32: 1238–1245.

11. Chuah, Y.J., Y. Zhang, Y. Wu, N. V. Menon, G.H. Goh, A.C. Lee, V. Chan, Y. Zhang, and Y. Kang. 2015. Combinatorial effect of substratum properties on mesenchymal stem cell sheet engineering and subsequent multi-lineage differentiation. Acta Biomater. .

12. Mullen, C.A., T.J. Vaughan, K.L. Billiar, and L.M. McNamara. 2015. The Effect of Substrate Stiffness, Thickness, and Cross-Linking Density on Osteogenic Cell Behavior. Biophys. J. 108: 1604–1612.

13. Lee, J., A.A. Abdeen, D. Zhang, and K. a. Kilian. 2013. Directing stem cell fate on hydrogel substrates by controlling cell geometry, matrix mechanics and adhesion ligand composition. Biomaterials. 34: 8140–8148.

14. Khani, M.-M., M. Tafazzoli-Shadpour, M. Rostami, H. Peirovi, and M. Janmaleki. 2014. Evaluation of mechanical properties of human mesenchymal stem cells during differentiation to smooth muscle cells. Ann. Biomed. Eng. 42: 1373–80.

15. González-Cruz, R.D., V.C. Fonseca, and E.M. Darling. 2012. Cellular mechanical properties reflect the differentiation potential of adipose-derived mesenchymal stem cells. Proc. Natl. Acad. Sci. U. S. A. 109: E1523–9.

16. Evans, E., and a Yeung. 1989. Apparent viscosity and cortical tension of blood granulocytes determined by micropipet aspiration. Biophys. J. 56: 151–160.

17. Hochmuth, R.M. 2000. Micropipette aspiration of living cells. J. Biomech. 33: 15–22.

18. Oh, M.-J., F. Kuhr, F. Byfield, and I. Levitan. 2012. Micropipette Aspiration of Substrate-attached Cells to Estimate Cell Stiffness. J. Vis. Exp. : e3886.

19. Shao, J.Y., and R.M. Hochmuth. 1996. Micropipette suction for measuring piconewton forces of adhesion and tether formation from neutrophil membranes. Biophys. J. 71: 2892–2901.

20. Tan, S.C.W., W.X. Pan, G. Ma, N. Cai, K.W. Leong, and K. Liao. 2008. Viscoelastic behaviour of human mesenchymal stem cells. BMC Cell Biol. 9: 40.

21. Pan, W., E. Petersen, N. Cai, G. Ma, J. Run Lee, Z. Feng, K. Liao, and K. Leong. 2005. Viscoelastic properties of human mesenchymal stem cells. Proc. 2005 IEEE Eng. Med. Biol. 27th Annu. Conf. Shanghai, China, Sept. 1-4, 2005. 5: 4854–4857.

22. Fletcher, D. a., and R.D. Mullins. 2010. Cell mechanics and the cytoskeleton. Nature. 463: 485–492.

23. Vichare, S., S. Sen, and M.M. Inamdar. 2014. Cellular mechanoadaptation to substrate mechanical properties: contributions of substrate stiffness and thickness to cell stiffness measurements using AFM. Soft Matter. 10: 1174–81.

24. Radmacher, M., M. Fritz, and C.M. Kacher. 1996. Measuring the viscoelastic properties of human platelets with the atomic force microscope. Biophys. J. 70: 556–567.

25. Dai, J., and M.P. Sheetz. 1995. Mechanical properties of neuronal growth cone membranes studied by tether formation with laser optical tweezers. Biophys. J. 68: 988–996.

26. Dao, M., C.T. Lim, and S. Suresh. 2003. Mechanics of the human red blood cell deformed by optical tweezers. J. Mech. Phys. Solids. 51: 2259–2280.

27. Lim, C.T., E.H. Zhou, and S.T. Quek. 2006. Mechanical models for living cells - A review. J. Biomech. 39: 195–216.

28. Song, F., and D. Ren. 2014. Stiffness of cross-linked poly(dimethylsiloxane) affects bacterial adhesion and antibiotic susceptibility of attached cells. Langmuir. 30: 10354–10362.

29. Chuah, Y.J., S. Kuddannaya, M.H.A. Lee, Y. Zhang, and Y. Kang. 2015. The effects of poly(dimethylsiloxane) surface silanization on the mesenchymal stem cell fate. Biomater. Sci. 3: 383–390.

30. Kuddannaya, S., Y.J. Chuah, M.H.A. Lee, N. V Menon, Y. Kang, and Y. Zhang. 2013. Surface chemical modification of poly(dimethylsiloxane) for the enhanced adhesion and proliferation of mesenchymal stem cells. ACS Appl. Mater. Interfaces. 5: 9777–84.

31. Lu, H., L. Guo, N. Kawazoe, T. Tateishi, and G. Chen. 2009. Effects of Poly(L-lysine), Poly(acrylic acid) and Poly(ethylene glycol) on the Adhesion, Proliferation and Chondrogenic Differentiation of Human Mesenchymal Stem Cells. J. Biomater. Sci. Polym. Ed. 20: 577–589.

32. Wu, M.-H. 2009. Simple poly(dimethylsiloxane) surface modification to control cell adhesion. Surf. Interface Anal. 41: 11–16.

33. Nojehdehian, H., F. Moztarzadeh, H. Baharvand, N.Z. Mehrjerdi, H. Nazarian, and M. Tahriri. 2010. Effect of poly-L-lysine coating on retinoic acid-loaded PLGA microspheres in the differentiation of carcinoma stem cells into neural cells. Int. J. Artif. Organs. 33: 721–30.

34. Merkel, R., R. Simson, D.A. Simson, M. Hohenadl, A. Boulbitch, E. Wallraff, and E. Sackmann. 2000. A micromechanic study of cell polarity and plasma membrane cell body coupling in Dictyostelium. Biophys. J. 79: 707–719.

35. Daniel, M., S. Gernald, and S. Winfield. 1975. Adhesion of cells to surfaces

coated with polylysine. J. Cell Biol. 66: 198–200.

36. Theret, D.P., M.J. Levesque, M. Sato, R.M. Nerem, and L.T. Wheeler. 1988. The application of a homogeneous half-space model in the analysis of endothelial cell micropipette measurements. J. Biomech. Eng. 110: 190–199.

37. Sato, M., D.P. Theret, L.T. Wheeler, N. Ohshima, and R.M. Nerem. 1990. Application of the micropipette technique to the measurement of cultured porcine aortic endothelial cell viscoelastic properties. J. Biomech. Eng. 112: 263–268.

38. Sartori, S., V. Chiono, C. Tonda-Turo, C. Mattu, and C. Gianluca. 2014. Biomimetic polyurethanes in nano and regenerative medicine. J. Mater. Chem. B. 2: 5128.

39. Macrí-Pellizzeri, L., B. Pelacho, A. Sancho, O. Iglesias-García, A.M. Simón-Yarza, M. Soriano-Navarro, S. González-Granero, J.M. García-Verdugo, E.M. De-Juan-Pardo, and F. Prosper. 2015. Substrate Stiffness and Composition Specifically Direct Differentiation of Induced Pluripotent Stem Cells. Tissue Eng. Part A. 21: 150318121612004.

40. Tee, S.-Y., A. Bausch, and P. Janmey. 2009. The mechanical cell. Curr. Biol. 19: 745–748.

41. Colley, H.E., G. Mishra, A.M. Scutt, and S.L. McArthur. 2009. Plasma polymer coatings to support mesenchymal stem cell adhesion, growth and differentiation on variable stiffness silicone elastomers. Plasma Process. Polym. 6: 831–839.

42. Berdyyeva, T.K., C.D. Woodworth, and I. Sokolov. 2005. Human epithelial cells increase their rigidity with ageing *in vitro* : direct measurements. Phys. Med. Biol. 50: 81–92.