Short title: Nuclear deformation of stem cells on micropillars

Subcellular cell geometry on micropillars regulates stem cell differentiation

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Abstract

While various material factors have been shown to influence cell behaviors, recent studies started to pay attention to the effects of some material cues on “subcellular” geometry of cells, such as self-deformation of cell nuclei. It is particularly interesting to examine whether a self deformation happens discontinuously like a first-order transition and whether subcellular geometry influences significantly the extent of stem cell differentiation. Herein we prepared a series of micropillar arrays of poly(lactide-*co*-glycolide) and discovered a first-order transition of nuclear shape as a function of micropillar height under the examined section area and interspacing of the pillars. The deformed state of the nuclei of mesenchymal stem cells (MSCs) was well maintained even after osteogenic or adipogenic induction for several days. The nuclear deformation on the micropillar arrays was accompanied with smaller projected areas of cells, but led to an enhanced osteogenesis and attenuated adipogenesis of the MSCs, which is different from the previously known relationship between morphology and

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differentiation of stem cells on flat substrates. Hence, the present study reveals that the geometry of cell nuclei may afford a new cue to regulate the lineage commitment of stem cells on the subcellular level.

Keywords: Cell nucleus; Nuclear deformation; Stem cell differentiation; Micropillar array; Poly(lactide-*co*-glycolide)

1. Introduction

One of the fundamental topics in the fields of biomaterials and regenerative medicine is cell-material interactions [1-5], which are dynamic and intricate. Cells are able to sense the external material features and integrate physiochemical signals, which eventually result in altered cell functions and gene expressions [6, 7]. In order to reveal cell-material interactions, various patterning techniques have been developed and applied in cell research [8-13]. Although there are increasing understandings on cell responses to material surfaces via cytoskeleton remodeling, relatively little attention has been paid to the responses of cell nuclei, such as nuclear deformation.

Nucleus is the largest as well as the stiffest organelle in a mammalian cell that makes a great contribution to the mechanical properties of the cell [14]. In a typical mechanotransduction pathway, a nucleus is connected to extracellular matrix (ECM) through the cytoskeleton to facilitate adaptive cell responses [15-17]. Most of genetic material is located in the nucleus for a eukaryote. Study of nuclear geometry and its influence on cell behaviors is thus crucial for the understanding of cell-material interactions on a subcellular level.

A few studies on deformation of cell nuclei such as nuclear elongation under transiently applied forces have been reported by techniques including micropipette aspiration [15, 18, 19], atomic force microscopy [19], microplate compression [14], microfluidic system [20] and so forth. A persistent nuclear elongation was achieved by adhesive microislands, which restricted aspect ratios (ARs) of cells to some extents [21-24]. Nanostructures were found to

generate nuclear deformation as well [25, 26]. Recently, groups of BX Cui and Y Cui discovered in their collaborative research that vertical nanopillar-induced nuclear deformation could be applied in probing *in situ* subcellular perturbation of nuclear mechanics [26]. Meanwhile, micropillar arrays were reported to deform nuclei severely [27-30]. The nuclear distortion of osteosarcoma-derived cell lines was observed on micropillars of poly(dimethyl siloxane) and poly(L-lactic acid) by Anselme and co-workers [27, 30]. Our group has also achieved the control of nuclear deformation of mesenchymal stem cells (MSCs) by applying different micropillar patterns [29]. Nevertheless, a very basic question has never been addressed: a nucleus changes continuously or discontinuously with material dimension, or in light of statistical physics, whether or not such a biological process obeys a first-order (discontinuous) transition. The present report will answer this question by a semi-quantitative observation of self-deformation of MSCs on micropillar arrays.

Another critical question is the effect of nuclear deformation on stem cell differentiation. Although it is known that the cells could survive and differentiate in spite of serious nuclear deformation [29], it remains to be explored whether or not the nuclear deformation affects the extents of stem cell differentiation. Various cues have been known to dictate fate decisions of stem cells in the past decade. Regardless of soluble factors, lineage-specific differentiation of stem cells is influenced by cell cues including cell shape [31-33], cell size [34, 35], cell-cell contact [35-37] and matrix cues containing stiffness [38-41], topography [42], functional groups [43], nanoscale distribution of ligands [44], and even molecular chirality on material surfaces [45]. This paper reports that the nuclear deformation remains after cell differentiation. Upon that, we hypothesized that a persistent nuclear deformation might be a

potential new cue to determine cell fate. This hypothesis was rationalized from the concept of chromosome territories to depict the non-random distribution of chromosomes and compartmentalization of nuclear architecture [46, 47]. Positioning of the chromosome territories is related to gene contents and exerts profound influence on gene expression [46]. In order to verify our hypothesis, we utilized micropillar arrays to study the nuclear deformation and the resulting stem cell differentiation, as well as the corresponding gene expression.

In this study, we examined osteogenic and adipogenic differentiations of MSCs on micropillar arrays of poly(lactide-*co*-glycolide) (PLGA). PLGA is a very useful biomedical polymer [48], and here its micropillar arrays were fabricated by molding from silicon micropit templates. Appropriate pillar width and pillar spacing were chosen to induce a severe self-deformation of cell nuclei. Under these size dimensions, MSCs derived from bone marrow of rats were cultured on micropillars with varied pillar heights to validate the existence of a critical pillar height for nuclear deformation. Stem cell differentiation was then induced in osteogenic or adipogenic medium. The degrees of differentiation were measured on high micropillars, with those on low micropillars and smooth PLGA films as controls. Based on these experiments, the effects of nuclear deformation on stem cell differentiation were explored and summarized.

2. Materials and methods

2.1. Preparation and characterization of micropillar patterns

PLGA (Purac Inc., Netherlands) composed of 85 wt% lactide (LA) and 15 wt% glycolide

(GA) units (PLGA85/15) with number-average molecular weight of 3.59×10^5 and molar mass dispersity of 1.72 was used to fabricate micropillars. The PLGA micropillar arrays were made by molding from a series of silicon wafer templates with micropits [29], and the micropits were ordered from Shanghai Institute of Microsystem and Information Technology, Chinese Academy of Sciences. A PLGA solution in dichloromethane (5 wt%) was casted onto these templates. The dried PLGA micropillars were carefully peeled off from the silicon wafers. As a control, a smooth PLGA film was also prepared by depositing the above PLGA solution on a smooth silicon wafer. The resulting samples were gold sputtered and observed under field-emission scanning electron microscope (FE-SEM, Ultra 55, Zeiss, Germany). The micropillar heights were then calculated using the software ImageJ 1.48v (National Institutes of Health, USA).

2.2. Isolation and culture of MSCs

MSCs were isolated from marrow of the tibiae and femora of neonatal Sprague Dawley (SD) rats [49]. The marrow was flushed out thoroughly using a sterile injection syringe filled with low-glucose Dulbecco's modified Eagle medium (DMEM, Gibco). Following the step of centrifuging, the supernatant was discarded, and the accumulated sediment was re-suspended in low-glucose DMEM supplemented with 10% fetal bovine serum (FBS, Gibco). MSCs of the second passage were used in the later experiments of cell adhesion and differentiation.

2.3. Staining of cells and morphological observations of nuclei

The PLGA films were sterilized by 75% alcohol for 45 min, fully rinsed by phosphate buffered saline (PBS) and then placed into 12-well tissue culture plates (TCPs). MSCs were seeded on the smooth and micropillared films at the density of about 2.6×10^3 cells per cm^2 .

After 24 h culture, cells on the PLGA films were rinsed gently with warm PBS twice, fixed with 4% paraformaldehyde for 10 min, and then permeabilized with 0.1% Triton X-100 in PBS for 10 min. The fixed MSCs were treated with 1 $\mu\text{g/ml}$ phalloidin-tetramethyl rhodamine B isothiocyanate (phalloidin-TRITC, Sigma) for 30 min to label filamentous actins (F-actins) followed with 4',6-diamidino-2-phenylindole (DAPI, Sigma) staining for 5 min to label nuclei. At last the samples were rinsed with Milli-Q water. The nuclei on micropillar arrays were observed under an inverted fluorescence microscope (Axiovert 200, Zeiss) equipped with a charge coupled device (CCD, AxioCam HRC, Zeiss).

Besides the examination of the deformed nuclei in the inverted microscope, we also reconstructed the vertical image stacks using a confocal microscope (LSM 710, Zeiss). In order to show both cell nuclei and micropillar arrays, we captured images using both standard detector (fluorescence or reflected mode) and transmitted detector (transmission mode).

2.4. Quantification of nuclear shape and statistics of its distribution

Shape index (SI) was introduced to quantify the shape of a nucleus. SI was defined as $4\pi S/l^2$, where the area (S) and perimeter (l) of each nucleus were measured using the software ImageJ 1.48v. Nuclei of at least 150 cells were measured on each group of micropillars.

In order to show distribution of SI, the probabilities of SI of cell nuclei with a step value of 0.05 were calculated from 0 to 1. According to the distribution profiles, we defined $\text{SI} < 0.8$ as the criterion of a deformed cell nucleus.

The fractions of deformed nuclei f were plotted as a function of micropillar height h . The data was fitted by a Boltzmann sigmoid. The equation can be, by adaptation from our previous determination of critical micelle concentration [50], written as

$$f = \frac{f_0 - f_\infty}{1 + e^{(h - h_{\text{critical}})/\delta}} + f_\infty$$

Here h_{critical} is the center of the Boltzmann sigmoid, giving the critical micropillar height for the deformation transition of cell nuclei; f_0 and f_∞ represent the asymptotic values of f at infinitely small and large h , respectively; δ reflects the deviation of h around h_{critical} .

The slope at the critical micropillar height was derived as

$$\left. \frac{df}{dh} \right|_{h=h_{\text{critical}}} = \frac{f_\infty - f_0}{4\delta}$$

Therefore, the transition zone in the Boltzmann sigmoid has a width of 4δ , with the onset of nuclear deformation occurs at $h_{\text{critical}} - 2\delta$ and the asymptotic end happens at $h_{\text{critical}} + 2\delta$, as schematically presented in Fig. S1.

2.5. Immunofluorescence staining of cells and confocal microscopic analysis

After 24 h culture in the growth medium, cells on the PLGA films with or without micropillar arrays were fixed, permeabilized and blocked with 5% bovine serum albumin (BSA). For immunostaining of vinculins, the cells were incubated with 1:100 diluted primary antibody of mouse monoclonal anti-vinculin (Sigma) at 4 °C overnight. After thoroughly rinsed with PBS, the cells were further treated with secondary antibody (Alexa Fluor 488-conjugated goat anti-mouse IgG, Invitrogen) diluted by 1:100, and incubated at room temperature for 2 h. Moreover, F-actins and nuclei were labeled as described in the above section. The samples were again observed under the inverted microscope or laser scanning confocal microscope. By using the software ImageJ 1.48v, individual cells were outlined to calculate projected area, AR and circularity of each cell. At least 400 cells were assessed on

each group of the PLGA films. Laser scanning confocal microscope (C2+, Nikon) was exploited to capture high-magnification fluorescence micrographs of cells on micropillar arrays.

For immunofluorescence staining of phosphorylated myosin light chain 9 (p-MYL9) on micropillar patterns, samples were fixed, permeabilized and blocked with BSA at room temperature for 30 min. Then the cells were incubated with the rabbit polyclonal p-MYL antibody (Santa Cruz) at 4 °C overnight. After rinsed with PBS, the cells were treated with anti-rabbit IgG-TRITC (Sigma) at room temperature the next day for 2 h. F-actins and nuclei were counterstained as mentioned above. Images were acquired with the epi-fluorescence microscope (Axiovert 200, Zeiss), keeping the intensity of excitation light and exposure time of photographing constant among all groups.

The 8-bit greyscale images of p-MYL9 and F-actin were exported using Zen software (Zeiss). Then the relative intensities (*RI*s) of p-MYL9 and F-actin of a cell were calculated as follows,

$$RI = \sum_{\text{cell}} (I - I_0) = S_{\text{cell}} (\langle I \rangle - I_0)$$

where S_{cell} is the pixel number of the projected area of a cell outlined by Image J, I_0 is the mean grey value of the substrate background nearby the cell, I is the grey value of p-MYL9 or F-actin in a pixel of the corresponding cell, and $\langle I \rangle$ is the mean grey level for each cell. The summation is done within the profile of a cell, and thus *RI* denotes an integrated fluorescence intensity for each cell. At least 150 cells were evaluated for each independent sample.

2.6. Osteogenic and adipogenic inductions of MSCs on PLGA micropillars

For osteogenic differentiation, the stem cells were seeded on micropillar arrays at a density of about 2.6×10^3 cells per cm^2 in the growth medium (low-glucose DMEM, 10% FBS). The medium was changed to the osteogenic induction medium after 6 h. The osteogenic medium was composed of high-glucose DMEM supplemented with FBS (10%, Gibco), ascorbic acid-2-phosphate (50 μM , Sigma), β -glycerophosphate (10 mM, Sigma) and dexamethasone (100 nM, Sigma). For adipogenic differentiation, the cells were first seeded at a density of about 1×10^4 cells per cm^2 and cultured in the growth medium for 6 h. Then the cells were exposed successively to the adipogenic induction medium and the adipogenic maintenance medium. The induction medium for adipogenesis was composed of high-glucose DMEM, FBS (10%), dexamethasone (1 μM), indomethacin (200 μM , Sigma), insulin (10 $\mu\text{g}/\text{ml}$, Sigma) and 3-isobutyl-1-methylxanthine (0.5 mM, Sigma); the maintenance medium contained FBS (10%) and insulin (10 $\mu\text{g}/\text{ml}$) in high-glucose DMEM. Both osteogenic and adipogenic induction periods lasted for 7 days, and the induction medium was renewed every 2-3 days. In order to inhibit cell proliferation, the cells were exposed to 0.5 $\mu\text{g}/\text{ml}$ aphidicolin (Sigma) throughout the culture period.

2.7. Analysis of osteogenesis and adipogenesis

Alkaline phosphatase (ALP) and oil droplets were employed as the biomarkers of osteoblasts and adipocytes, respectively. Osteogenically induced cells were fixed in 4% paraformaldehyde for 10 min and then stained by Fast Blue RR/naphthol (Sigma) for ALP according to the manufacturer's protocol. Afterwards, DAPI was added to mark the nuclei. To examine the efficacy of adipogenesis, cells were rinsed by Milli-Q water and 60%

isopropanol successively after fixation with 4% paraformaldehyde, treated with fresh Oil Red O (Sigma) solution in 60% isopropanol for 15 min and rinsed by Milli-Q water again. Cell nuclei were also labeled by DAPI. The cells containing oil droplets were identified as adipocytes. The stained cells on micropillar arrays were observed in the inverted microscope mounted with a CCD. Micrographs were captured for later statistical analysis.

To assess ALP activity quantitatively, cell lysis was carried out in lysis buffer containing 1% Triton X-100. After centrifugation at 4 °C for 15 min, the supernatant was aspirated and further used for measurement of the ALP activity. The total ALP activity was quantified with Alkaline Phosphatase Assay Kit (Beyotime) and the total protein content was measured with BCA Protein Assay Kit (Beyotime). The ALP activity was thus calculated and defined as nmol per mg protein per assay time. As for adipogenic induction, the probabilities of adipogenesis of cells on different micropillar arrays were calculated by counting the fractions of cells that committed to an adipogenic lineage.

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

After 7 d of induction (samples were in triplicates), the total RNA of the cells was extracted using the MagneSil mini-Isolation System (Promega) according to vendor's protocol. The concentration and purity of the extracted RNA were tested by Nanodrop 2000 (Thermo Scientific). The RNA samples were then reversely transcribed into the first-strand cDNA by applying the PrimeScript RT reagent Kit with gDNA Eraser (TaKaRa). qRT-PCR was carried out with Rotor-Gene SYBR Green Kit (Qiagen) to analyze the expressions of ALP, Runt-related transcription factor 2 (Runx2), lipoprotein lipase (LPL) and peroxisome

proliferator-activated receptor γ (PPAR γ) genes using Rotor-Gene 2plex System (Qiagen). We employed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the house-keeping gene. Data analysis was performed using the $2^{-\Delta\Delta C_t}$ method. The expressions of target genes were normalized to the GAPDH expression. The results were further normalized to the mean values of the corresponding groups of smooth films.

2.9. Statistical analysis

Independent experiments ($n = 4$) were averaged during the analysis of the osteogenic and adipogenic differentiations. For the cell adhesion parameters, p-MYL9 and F-actin expressions, three samples ($n = 3$) were averaged, and standard deviations were calculated. One-way analysis of variance (One-way ANOVA) was carried out to analyze the results between different groups using SPSS 16.0 software (SPSS Inc., Chicago, USA). We considered a difference as significant when the p value was less than 0.05.

3. Results

3.1. Self-deformation of nuclei of MSCs on micropillar arrays

A series of PLGA micropillar arrays with different micropillar heights were obtained, with some side-view FE-SEM images shown in the upper row in Fig. 1A. The side length of the square sections of the fabricated micropillars and the spacing between the micropillars were about 3 μm and 6 μm , respectively, as shown in a top-view FE-SEM image in Fig. S2. The nuclear deformation with an increase of micropillar height is demonstrated from some fluorescence micrographs in the lower row in Fig. 1A. Higher micropillars led to more severe compliance of the shape of cell nuclei, which were significantly influenced by the position of

those micropillars. It is worth mentioning that the PLGA micropillars of the dimensions we examined are quite rigid and the spreading of cells did not bend the pillars noticeably in our experiments.

Fig. 1. Self-deformation of cell nuclei on micropillar arrays. (A) SEM images of smooth and micropillared PLGA films with indicated pillar heights (upper row) and the fluorescence micrographs to show representative nuclei of cells (lower row). The micropillars in the lower row were artificially marked based on corresponding bright-field images. (B) Confocal microscopic analysis of nuclear deformation of MSCs after 24 h culture on 6.4 μm micropillars. The middle column shows the merged images of the fluorescence mode (reflected light imaging) captured by the standard detector to show cell nuclei (blue) and F-actins (red), and the transmitted light imaging mode captured by the transmitted detector to show micropillar arrays (grey). At the cost of low intensity of F-actins, a low gain was used in the transmitted detecting to make a “black” background in order to match with the corresponding fluorescence micrograph in the merged image. The cross sections of the three-dimensional cell nuclei configuration by confocal

microscopy are schematically presented on the right side. A scan along the Z direction was used in capture of fluorescence micrographs. While images of the XY plane along the below yellow lines are shown on the upper row, the reconstructed images of the XZ plane along the above yellow lines are displayed in the bottom row.

We also used confocal microscopic imaging to observe the three-dimensional configuration of the nuclei, as demonstrated by the case of micropillars of 6.4 μm height. The schematic illustrations on the right side of Fig. 1B presented the section analysis of the cell nuclei configuration via confocal Z-stack microscopic reconstructions. Substrate and fluorescence images were merged to show the deformed nuclei among the micropillars (Fig. 1B). The top-down sections unambiguously confirmed that the nuclei deformed among the pillars. The nuclear deformation was two-dimensionally visible.

3.2. Transition from no significant deformation to serious self-deformation of cell nuclei with the increase of micropillar height

To examine the dependence of nuclear deformation on micropillar height under the given section area and interspacing of pillars, SI calculated from area and perimeter of a nucleus was employed to quantify the extent of nuclear deformation, where 1 stands for a perfect circle. Some calculated values along with the fluorescence micrographs are shown in Fig. S3. We defined the nucleus with an index less than 0.8 as a deformed nucleus. This criterion is based upon the distribution of the index. The percentages of deformed nuclei on smooth films and micropillars with different pillar heights are plotted in Fig. 2A. Hardly any nuclei deformed on smooth films and 0.8 μm micropillars, where nuclei appeared in circular or oval shapes. In contrast, an approximately half of the nuclei distorted into the space between the pillars when the micropillar height grew to 3.2 μm . Deformed nuclei were predominant in the

other three groups of higher micropillars.

The data points in Fig. 2A were well fitted by Boltzmann function with the equation described in the section of Materials and methods. The profile of this function is schematically presented in Fig. S1. The Boltzmann fitting resulted in the transition point at $3.13 \mu\text{m}$ with a transition zone of $1.66 \mu\text{m}$ in width. As a result, the nuclei started to deform at $2.30 \mu\text{m}$; the change took place most abruptly with micropillar height at $h_{\text{critical}} = 3.13 \mu\text{m}$; and the fraction of nuclear deformation reached asymptotic after $h > 3.96 \mu\text{m}$.

Fig. 2. First-order transition from non-deformation to significant nuclear deformation with increasing micropillar height. (A) The average fraction of deformed nuclei as a function of micropillar height after culturing the stem cells for 24 h. Deformed nuclei were defined as those of shape index (SI) less than 0.8. $SI = 4\pi S/l^2$, where S and l denote area and perimeter of a cell nucleus, respectively. The data was fitted by Boltzmann sigmoid, resulting in the indicated critical height h_{critical} . **(B)** Distributions of SI of cell nuclei on PLGA micropillars of indicated heights as well as on smooth PLGA films. A bimodal distribution reflects a first-order phase transition near the critical pillar height.

We also carried out statistics of SI distributions of nuclei. The SIs exhibited a bimodal distribution on $3.2 \mu\text{m}$ micropillars, while SI distributions of nuclei on all other films were unimodal (Fig. 2B). For films of 4.6 , 5.3 and $6.4 \mu\text{m}$ micropillars, peak positions of each

curve located at less than 0.8. Meanwhile, the population distributions of SIs on smooth and 0.8 μm micropillared films were relatively higher and narrower with peak positions greater than 0.8. The double peaks of the bimodal curve were located on both sides of SI 0.8, which clearly indicates that the change of nuclear deformation in this study happens not continuously but obeys a first-order transition.

3.3. Adhesion of MSCs with deformed nuclei on micropillar arrays

For further investigation of the effects of nuclear deformation on cell adhesion and differentiation, behaviors of cells on micropillar arrays with pillar heights of 0.8, 4.6 and 6.4 μm as well as smooth films were examined. Representative fluorescence micrographs of MSCs after 24 h of culture are presented in Fig. 3A. For cells on 0.8 μm micropillars and smooth films, prominent and highly-organized F-actins were observed; mature focal adhesions were formed as indicated by clustered vinculins on the cell periphery; and the nuclei showed natural shapes. With the increase of pillar height to 4.6 μm or larger, focal adhesions and actin cytoskeletons were densely distributed around the micropillars and became obscure in the 2D images.

The corresponding adhesion parameters such as projected area, AR and cell circularity were also quantified, with the results shown in Fig. 3B. There were no significant differences in cell projected area between smooth films and 0.8 μm micropillars as well as between 4.6 μm and 6.4 μm groups. Deformation of cell nuclei occurred on micropillar arrays with pillar heights of 4.6 or 6.4 μm and meanwhile the projected areas of those cells declined.

AR, as an indicator of cell shape, was defined as the major axis divided by the minor axis of the cell contour, which was determined from the cell profile outlined with ImageJ software.

The means of AR in Fig. 3B increased from smooth films to micropillars of 4.6 μm height, indicating that those micropillars enabled cell elongation along the ordered micropillars. Cell circularity, another important cell shape parameter, was defined as projected areas multiplied by 4π and divided by the square of perimeter, with 1.0 standing for a perfect circle. The decrease in cell circularity with the pillar height may be attributed to the cell elongation and more pseudopodia. The frequency distributions of projected areas and cell shape indicators of MSCs are shown in Fig. S4.

Fig. 3. Adhesion of MSCs cultured on micropillared and smooth PLGA films after 24 h. (A) Typical confocal fluorescence micrographs, where nuclei were stained in blue by DAPI, F-actins in red by phalloidin-TRITC, and vinculin in green by a monoclonal anti-vinculin antibody and a fluorescent secondary antibody. The insets are solely for nuclei and micropillars. (B) Statistical results of cell adhesion parameters including projected area, aspect ratio and circularity per cell. Mean values and standard deviations from three independent groups are presented. Marked in the figure are significant differences with “*” ($p < 0.05$), “**” ($p < 0.01$) and “***” ($p < 0.001$).

3.4. Maintenance of nuclear deformation of stem cells even after differentiation induction

In order to investigate the effects of nuclear deformation on stem cell differentiation, MSCs on the indicated micropillared and smooth films were cultured in the osteogenic or adipogenic induction medium for 7 d following in the growth medium for 24 h. Since the whole induction period lasted for 7 d, a critical question was raised: whether the deformed nuclei would recover or not on micropillar arrays (Fig. 4A). Anselme's group observed that cells (an osteoprogenitor cell line named HOP) exhibited deformed nuclei on micropillars at short time points but the deformation was recovered to the natural nuclear shape after 2 days [28]. We confirmed the recovery of some non-stem cells under similar conditions. In the present study of differentiation of stem cells with nuclei severely deformed, it is thus crucial to verify that a majority of nuclei of our MSCs maintain the deformed status throughout the experimental period for several days and thus to make sure that the nuclear deformation can persistently exert influence on cell differentiation.

Fig. 4. Maintenance of significant self-deformation of stem cells on micropillar arrays even after induction. (A) Schematic presentation of a critical question whether the self-deformation of cell nuclei would recover or not on micropillar arrays after a long cell induction period. (B) Representative fluorescence micrographs of cell nuclei after induction for 7 d, demonstrating the persistence of the nuclear deformation on the present conditions.

After 7 days of osteogenic or adipogenic induction of MSCs, the nuclei were stained and examined under a fluorescence microscope. The deformation of nuclei was well maintained, with some representative nuclei presented in Fig. 4B. A persistent nuclear deformation might be caused by the change of nuclear mechanics during differentiation based on previous observations of cells on flat substrates [51]. The present observations demonstrated that nuclear deformation of MSCs on micropillars remained also during the induction period. It is not hard to understand that stem cells have relatively soft nuclei due to less dense lamina.

3.5. Osteogenic and adipogenic inductions of MSCs on micropillar arrays

We also carried out differentiation experiments towards osteogenesis and adipogenesis. As shown in Fig. 5A, ALP and oil droplets, which are indicators of osteoblasts and

adipocytes respectively, were positively expressed. The generated oil droplets resided mostly between the micropillars of height 4.6 or 6.4 μm , while the appearance of oil droplets showed no relevance to the position of the underneath micropillars of height 0.8 μm . The corresponding cell nuclei are presented in Fig. S5. Besides, quantitative estimation after sole osteogenic or adipogenic induction for 7 d was conducted, with the results shown in Fig. 5B. The ALP activity on 4.6 and 6.4 μm micropillars accompanied with nuclear deformation was higher than that of 0.8 μm and smooth group. As opposed to this, the adipogenic fractions reduced on 4.6 and 6.4 μm micropillars compared to the other groups.

Fig. 5. Differentiation of MSCs on micropillared and smooth PLGA films. (A) Representative bright-field micrographs of cells after osteogenic or adipogenic induction. The upper row demonstrates that

ALP, as a biomarker for osteogenesis of MSCs, was stained in blue by Fast Blue RR; and the lower row displays that oil droplets, as biomarkers for adipogenesis of cells, were stained in red by Oil Red O. **(B)** Statistics of ALP activity and adipogenic fraction of MSCs after induction for 7 d. Means and standard deviations from four independent groups are shown. Marked in the figure are significant differences with “**” ($p < 0.01$) and “***” ($p < 0.001$).

3.6. Gene expressions of induced cells on micropillar arrays

Relative gene expressions on the micropillared and smooth films were additionally quantified using qRT-PCR. We selected ALP and Runx2 as characteristic genes for osteogenesis, LPL and PPAR γ as characteristic genes for adipogenesis, and GAPDH as the house-keeping gene. Pre-designed primer sequences for these genes are depicted in Fig. 6A. The measurements of gene expressions (Fig. 6B) agreed with the measured ALP activity and the statistics of adipogenic fraction (Fig. 5B).

A

Target gene	Forward primer (5'-3')	Reverse primer (5'-3')
ALP	ATGGTAACGGGCTGGCTACA	AGTTCTGCTCATGGACGCCGT
Runx2	AGATGGGACTGTGGTTACCG	GGACCGTCCACTGTCACTTT
LPL	ATGGATGGACGGTGACAGGA	CCAAGACTGTACCCTAAGAGGTG
PPAR _γ	CCTTTACCACGGTTGATTCTC	GGCTCTACTTTGATCGCACTTT
GAPDH	GCTCTCTGCTCCTCCCTGTTCTAG	TGGTAACCAGGCGTCCGAT

B

Fig. 6. Relative gene expression of osteogenically and adipogenically induced MSCs on micropillared and smooth films. (A) The pre-designed primers of target genes. ALP and Runx2 represent characteristic genes for osteogenesis, whereas LPL and PPAR_γ denote characteristic genes for adipogenesis. GAPDH serves as a house-keeping gene. (B) qRT-PCR detection of target genes after 7 d induction of MSCs on micropillars of indicated heights as well as on smooth PLGA films. The gene expressions on micropillared films were normalized to the mean values on the smooth films. Marked in the figure are significant differences with “***” ($p < 0.001$).

3.7. Cytoskeletal tension of MSCs with deformed nuclei on micropillar arrays

Cytoskeletal tension can be characterized by phosphorylated myosins [31, 38] and F-actins [40]. A myosin II molecule is a dimer comprised of two identical myosin molecules holding

together by their tails. The head ones interact with actin filaments, and they made up the major contractile proteins. Myosin light chain 9 (MYL9), which is one of the regulatory myosin light chains, is widely expressed in different tissues [52]. In our study, p-MYL9 was labeled to show intracellular tension. Representative fluorescence micrographs of MSCs with p-MYL9 and F-actin stained are presented in Fig. 7A. The color images of p-MYL9 and F-actin were converted to 8-bit greyscale ones using Zen software (Zeiss), and then the integrated fluorescence intensities of p-MYL9 and F-actin per cell were semi-quantitatively analyzed according to the protocol in the section of Materials and methods.

The integrated fluorescence intensity of p-MYL9 per cell on 4.6 or 6.4 μm micropillared films with nuclear deformation was relatively lower than that on smooth or 0.8 μm micropillared films according to Fig. 7B. The expressions of F-actins exhibited a similar tendency. Since the expression levels of phosphorylated myosins and F-actins partially reflect cell tension, Fig. 7 illustrated that cell tension decreased on relatively high micropillar arrays. The frequency distributions of integrated fluorescence intensity of p-MYL9 and F-actin per cell are displayed in Fig. S6. The peak values in the distribution graphs further confirmed the trend of cell tension.

Fig. 7. Fluorescent indication of cell tension on micropillar arrays. (A) Typical fluorescence micrographs of single stem cells on the indicated surfaces cultured in the growth medium for 24 h. Green: p-MYL9, Red: F-actin, blue: nucleus. (B) Integrated fluorescence intensity of p-MYL9 and F-actin normalized by the mean values of the group of 4.6 μm micropillars. At least three samples were analyzed for each group, and for each sample at least 150 cells were analyzed. Marked in the figure are significant

differences with “***” ($p < 0.001$).

4. Discussion

Eukaryocytes respond to the surrounding topographic features [6, 53-55]. Therefore, substrate topography can be used to regulate cell behaviors in tissue engineering and regenerative medicine [56-58]. Lineage commitment of stem cells on substrate topography has attracted much attention in the recent decades [42, 59-61]. Herein, severe nuclear deformation of MSCs was generated by a micropillar array, which acted as a non-invasive platform. The present study is aimed at examination of a critical transition from no significant deformation to severe self-deformation of cell nuclei induced by the topographical cue, and whether or not the nuclear deformation is able to regulate stem cell differentiation as a subcellular geometric cue.

4.1. *First-order transition of nuclear deformation as a function of micropillar height*

We prepared a series of micropillar arrays with different pillar heights. Significant self-deformation of nuclei of MSCs was observed in the cases of high micropillars. The top-down cross section images from confocal microscopy demonstrated that the nucleus deformed amazingly among the micropillars (Fig. 1B). As the pillar height increased, the nuclear shape changed from a circle or an ellipse to a deformed shape such as a cross.

We further checked out the critical micropillar height as a transition from no significant nuclear deformation to a significant self-deformation of nuclei. The percentages of deformed nuclei of cells on smooth films and micropillar arrays with the pillar height of 0.8, 3.2, 4.6, 5.3 and 6.4 μm were well fitted by a smooth sigmoid curve described by Boltzmann function,

as shown in Fig. 2A, resulting in the transition height at 3.13 μm . In physics, a bimodal distribution is a characteristic of the first-order phase transition [62, 63]. Here in such a biological process, the frequency of SI appeared in a bimodal distribution at the pillar height of 3.2 μm (Fig. 2B), reminiscent of a first-order phase transition.

We also tried to understand the physical meaning behind the critical pillar height. When nuclear deformation occurred, the cytoskeleton deformed simultaneously. In the cases of adequate pillar heights, the resultant significant insertion of the cytoskeleton into the space between micropillars might enable the self-deformation of nuclei.

The critical pillar height for nuclear deformation was semi-quantitatively estimated as follows. First, the average volume of MSCs was obtained based on the statistics of suspended cells. According to the measurement with automated handheld cytometer (Scepter 2.0, Millipore), the average volume of MSCs V approximated 3054 μm^3 . Moreover, the projected area of a cell in the case of nuclear deformation S was about 1320 μm^2 , as shown in Fig. 3B. The micropillars actually took up some space beneath the projected area, and the correction factor m , namely the ratio of the area without micropillars to the total area, equaled to 8/9 approximately, as schematically presented in Fig. S7. Hence, the critical pillar height was estimated as

$$h_{\text{critical}} \geq h = V/(mS) = 2.6 \mu\text{m}$$

The visualized nuclear deformation in our experiments indicates those piercing-like nuclei through the pillars, which are thus visible two dimensionally. In the cases of insufficiently high micropillars, some mild nuclear distortions may happen, but cannot be

observed in a common fluorescence microscope. Smooth films, acted as the control group, can be viewed as a special kind of micropillared films in which the pillar height is zero.

The critical micropillar height in a cell might be dependent on h_0 , the “natural” nuclear height of the cell. We then estimated the nuclear height of a cell spreading well on a smooth film. Taking a representative and well spread cell for example (Fig. S8), the confocal cross section images along the Z direction from top to bottom revealed that the nuclear height was about 2.8 μm and the cell height was about 3.5 μm when a cell adhered to the smooth film. In order to guarantee the piercing-like nuclear deformation, the desirable micropillar height should be higher than the most probable nuclear height of cells on smooth films, namely about 2.8 μm . The inferred conclusion here is quite close to our experimental observations of MSCs on the micropillar arrays. The supposed relationship between micropillar height and piercing-like nuclear deformation is schematically illustrated in Fig. 8A. We use the term “piercing-like” to describe such a nuclear deformation to emphasize the so severe deformation that cell nuclei fully invade into the space among the micropillars and were significantly squeezed by the micropillars.

4.2. Unusual trends of stem cell differentiation with adhesion parameters for cells with deformed nuclei

MSCs on micropillar arrays with varied micropillar heights differed in adhesion parameters, namely projected area, AR and cell circularity (Fig. 3). We hypothesized that the lineage specification of stem cells might be influenced by nuclear deformation in the cases of high micropillars. Along with the nuclear deformation, projected areas of cells decreased and cell ARs increased. It is well known that a decreased area favors adipogenesis and an

increased spreading is beneficial for osteogenesis [34, 35]; our group has, based on two-dimensional micropatterned surfaces with cell adhesion contrast, found that the optimal AR is about 1 for adipogenesis and 2 for osteogenesis [32].

These regulations on flat substrates are, however, not reflected for cells with significant nuclear deformation on micropillars as a quasi-three dimensional microenvironment of a material. Our differentiation results revealed that osteogenesis was enhanced and adipogenesis was alleviated on the higher micropillar arrays with nuclear deformation (Fig. 5B), although in these cases the projected areas of cells were not enlarged and cell ARs were significantly larger than 2 (Fig. 3B). Hence, the adhesion parameters alone cannot explain the differentiation phenomenon here.

Cytoskeleton actively takes part in the signal transduction of a cell responding to a substrate. It transmits signals from ECM to the nucleus and influences gene expressions [15, 64]. Cytoskeletal tension is thus an important factor in cell fate decision [34]. The RhoA/Rho-associated protein kinase (ROCK) signal pathway may be involved in the micropillar induced self-deformation of cell nuclei, since the addition of Y27632, an ROCK inhibitor, led to a significantly less deformation of nuclei of MSCs, as indicated by the confocal fluorescence micrographs shown in Supplementary Fig. S9.

Nevertheless, cell tension might not necessarily dominate the stem cell differentiation in the case of nuclear deformation on micropillars. Although a smaller projected area of cells generally means lower cell tension and thus weaker osteogenesis, the higher micropillars and the occurrence of nuclear deformation in this study complicated the underlying effect. A

further study on cell tension was performed to speculate whether or not cell tension mostly influenced lineage commitment of stem cells in this research system. The contractile forces produced by F-actin cables together with the associated myosin motors account usually for the generation of mechanical tension in cells. In our experiments, less spread MSCs on 4.6 or 6.4 μm micropillared films exhibited lower cell tension (Fig. 7B), but favored osteogenesis (Fig. 5B); more spread MSCs with higher cell tension on smooth or 0.8 μm micropillared films (Fig. 7B) showed better adipogenesis (Fig. 5B). It seems that cytoskeletal tension is not the only conclusive factor that decides fate of these stem cells.

Then what is the dominant factor behind this special phenomenon? Our attention is shifted to the severe nuclear deformation on higher micropillars, especially the possibility that the severe change of nuclear shape influences the gene expressions and regulates stem cell differentiation. Our experimental results positively support our hypothesis, and the gene expressions of osteoblasts and adipocytes with deformed nuclei (Fig. 6) were consistent with the cell-staining results (Fig. 5). The characteristic genes of osteoblasts were up-regulated and the characteristic genes of adipocytes were down-regulated on micropillar arrays of height 4.6 or 6.4 μm with the nuclear deformation.

Chromatin architecture and chromatin condensation make differences in a series of fundamental cellular processes including mRNA transcription, DNA replication, recombination, repair, along with the transition between interphase and mitosis [65]. For instance, gene expressions may be affected in the process of mRNA transcription by exposure or obscuration of molecular binding sites [66]. Healy group reported that changing nuclear shape altered gene expression and protein synthesis of primary rat bone cells on multidomain

patterns [67]. Gabriele group revealed that nuclear deformation led to a drastic condensation of chromatin and lower cell proliferation rate accompanied with elongation of endothelial cells [22]. Based on those studies, it is reasonable that appropriate micropillar arrays with serious self-deformation of nuclei might lead to different gene expressions of stem cells.

As a part and parcel in mechanotransduction, cell nucleus is, besides cytoskeleton, another main contributor to the mechanical properties of the whole cell under the external forces [14]. The nucleus may be constructed as a tensegrity like the whole cell model, since tensional and integrated structure provides mechanical stability [68], which is pivotal for cell behaviors including migration [69, 70], proliferation [71] and polarization [69]. Dahl et al. believed that the transmission of mechanical forces to the nucleus and the triggered nucleus deformation relied on the mechanical contribution of the nucleus as well as its physical link to the cytoskeleton [66]. Besides, external forces could lead to the conformational changes of DNA or chromatin structure, and then the changes in transcriptional activity. The effects of topography-induced changes of morphology of fibroblasts on territory positioning of chromosomes and related gene expressions were examined by Dalby and his colleagues [57]. In general, transcriptionally active euchromatin is usually located more centrally within the nucleus, while heterochromatic sequences correlated with the nucleoli are rarely expressed on the periphery of the nucleus [72].

In our study, not only topography-induced cytoskeleton reorganization but also nuclear deformation happened at higher micropillars. The changes of cell tension and cell adhesion parameters in concert with topography are insufficient to interpret the significant change of stem cell differentiation. As for the subcellular geometric cue, it should be of no surprise that

the dramatic deformation of nucleus, which connected ECM through cytoskeleton, could directly or indirectly lead to the changes of chromatin structure and transcriptional activity. Hence, nuclear deformation is one of the potential cell cues to regulate stem cell fate apart from some known cues like cell shape, cell size and cell-cell contact. The deformed nuclei may affect chromosomal positioning, thus influencing gene expression patterns and differentiation programs of MSCs.

The effects of nuclear deformation on differentiation of stem cells cultured on arrays of high micropillars are schematically presented in Fig. 8, along with the supposed relationship between pillar height and piercing-like nuclear deformation. Nuclear deformation in our study referred to the deformation of a nucleus like piercing through the pillars and thus visualized two dimensionally. In Fig. 8A, we hypothesized that under appropriate pillar width and pillar spacing, the visualization of nuclear deformation happens in the context that the pillar height appears higher than the nucleus height of a well spreading cell on the smooth films. Nuclear deformation on arrays of higher micropillars regulates, outweighing cell adhesion and inherent cell tension, differentiation of MSCs towards more osteogenesis and less adipogenesis (Fig. 8B). Nonetheless, it is not clear how nuclear deformation affects the gene expressions in this process. The underlying transduction pathways remain to be further investigated.

Fig. 8. Schematic illustrations of critical dimension for significant nuclear deformation of cells on micropillar arrays and the effect of the nuclear deformation on stem cell differentiation. (A) Supposed relationship between micropillar height and piercing-like nuclear deformation. A piercing-like nuclear deformation and thus a visible deformation from a top-down view happen when the pillar height is higher than the nuclear height of a spread cell. (B) Schematic effects of nuclear deformation on differentiation of MSCs towards osteoblasts and adipocytes on micropillar arrays. The effect of nuclear deformation as a kind of subcellular cell geometry cue to regulate stem cell differentiation.

5. Conclusions

PLGA micropillars were fabricated by molding from silicon wafers, and shapes of nuclei of MSCs were controlled and quantified on arrays with a series of micropillar height. A first-order transition of cell nuclei from no significant deformation to significant self-deformation was discovered, and a double-peak distribution was observed for the shape indexes of cell nuclei around the critical pillar height under appropriate section area and interspacing of micropillars. The average fraction of deformed nuclei versus micropillar

height obeyed Boltzmann sigmoid. Stem cell adhesion and differentiation were conducted on micropillar arrays above and below the critical pillar height. The self-deformation of cell nuclei in the cases of high micropillars maintained even after induction of MSCs. Such a persistent nuclear deformation on material surfaces influenced the extents of differentiation of MSCs, and the effects were found to be dependent upon the type of committed lineages. Osteogenic differentiation was enhanced and adipogenic differentiation was alleviated on micropillared arrays with significant self-deformation of cell nuclei, in spite of smaller projected areas and lower cell tension of MSCs. Hence, the present study reveals that nuclear deformation affords a new subcellular geometric cue to influence differentiation of stem cells, for significant self-deformation of cell nuclei on micropillars is, as an intracellular signal modulator for lineage commitment of stem cells, a plausible mechanism for topography induced regulation of cell differentiation.

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