Chromatin Reprogramming via Contact Guidance-Induced Nuclear Deformation Promotes Stem Cell Differentiation

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Abstract

Efficient manipulation of cell fate is important for regenerative engineering applications. Lineage-specific differentiation of stem cells is particularly challenging due to their inherent plasticity. Engineered topographies may alter cellular plasticity through contact guidance. However, the ability to rationally design topographies to regulate phenotypic outcomes has been hindered in part by the lack of tools to quantify nanoscale chromatin structure reorganization in live cells. Herein we use micropillars, molecular, and nanostructural quantification tools to investigate how nuclear morphology in human mesenchymal stem cells (hMSCs) affects chromatin conformation and osteogenic differentiation. We show that micropillar-induced contact guidance is transduced via the cytoskeleton and impacts nuclear architecture, lamin A/C multimerization, histone modifications, and the 3-D conformation of chromatin within packing domains, a key regulator of transcriptional responsiveness. Micropillars repressed expression of genes associated with developmental processes and enhanced lineage-specific responsiveness, thereby decreasing cell plasticity and off-target differentiation, and facilitating osteogenic differentiation of hMSCs. Altogether, these findings reveal that chromatin reprogramming through contact guidance-induced nuclear deformation can be an efficient way to manipulate cell fate.

Background

Nuclear morphology is regulated by nuclear structure components such as lamins and chromatin, as well as cytoskeletal proteins.¹ Although not fully understood, studies have revealed that mammalian cells can modulate their nuclear morphology to adapt and acclimate to their microenvironments through the mechanotransduction process.²⁻⁵ Usually, the nucleus is considered to be of spherical or ovoid shape, which is true for many types of cells. However, severe changes in nuclear morphology are also observed in various physiological processes such as malignant cell invasion,⁶ smooth muscle cell contraction,⁷ stem cell homing,⁸ and embryo development.⁹ As a cellular mechanosensor, changes in nuclear morphology are considered to directly affect chromatin reprogramming and genome functions that determine cell fate.¹ Contact guidance-induced nuclear deformation, similar to what occurs *in vivo*, can be reproduced in cells cultured on micro- and nano-pillar substrates.^{10,11} The resultant nuclear deformation has been shown to affect phenotypic outcomes in stem cells such as proliferation and differentiation.¹²⁻¹⁴ Maximizing cell differentiation in cell culture and at tissue-implant interfaces is a major goal in many fields including tissue and regenerative engineering, surgery, and biological sciences.¹⁵ However, the relationship between the nuclear deformation induced by such topographies and phenotypic outcomes remains elusive. Additionally, direct evidence for contact guidance-induced chromatin reprogramming in live cells during such processes is limited.¹⁶

In this study, nuclear morphology changes were induced via contact guidance on micropillars to investigate their influence on osteogenic differentiation of human mesenchymal stem cells (hMSCs). We hypothesized that mechanical constriction of the nucleus will lead to chromatin reprogramming, and as a consequence, modulate the transcriptional plasticity in stem cells to improve the efficiency of lineage-specific differentiation. Previously, we have demonstrated that the physical structure of chromatin packing regulates genome-wide transcriptional patterns by altering the kinetics of transcriptional reaction through macromolecular crowding-mediated effects exerted by chromatin density that is inter-related with the local chromatin nanoenvironment.¹⁷ We uncovered that chromatin exhibits length-scale invariant chromatin packing scaling behavior within chromatin packing domains, with sizes on the order of 100s of nm.¹⁸ Specifically, we have identified the chromatin packing density scaling of packing domains, D , as an important statistical descriptor of chromatin behavior and transcriptional plasticity.

From a polymer physics definition, D defines the power-law scaling relationship between the genomic length of a polymer and the space it occupies in three-dimensional space. Furthermore, combining the molecular and physical regulators of transcription, the chromatin packing macromolecular crowding (CPMC) model predicts the effect of average packing domain D on global patterns of gene transcription. The model shows that an increase in D increases both the accessible surface area of chromatin, which determines the probability of genes being accessible to transcription factors, and the heterogeneity of crowding conditions within a given transcriptional interaction volume.¹⁷ Therefore, given that D is one of the major predictors of global gene expression, we hypothesized that the differentiation outcomes in hMSCs can be modulated by potentially altering this physical property of chromatin. To address this hypothesis, we integrated surface topography engineering of biomaterials, nanoscale imaging, cell, and molecular biology to investigate the influence of severe nuclear deformation in hMSCs on chromatin reprogramming and transcription, as well as osteogenic differentiation.

Results

Micropillars Manipulate Nuclear Morphology. In order to study the implications of mechanical constriction of the nucleus on stem cell fate, micropillar structures were fabricated using methacrylated poly (1,8-octanediol-co-citrate) (mPOC) via contact printing (Fig. S1A). A variety of parameters including pillar size, shape, and space were controlled to investigate their effects on nuclear morphology (Fig. S1B). All the pillar structures had a height of 8 μm which is sufficient to cause deformation in the nucleus.¹³ Nuclear shape index (NSI) was analyzed to quantify the effect of micropillars on nuclear morphology.¹⁹ We found that spacing of micropillars had the most obvious influence on nuclear morphology as decreasing the spacing resulted in more severe deformation of the nucleus (Fig. S1C, D). The shape of micropillars also had a significant influence on nuclear morphology due to the differences in the curvature of the microfeatures. Herein, we found that micro-square pillars with a size and spacing of 5 x 5 μm had the most significant effect on the deformation of hMSC nuclei, which was used for the following studies (Fig. 1A-D).

Cytoskeleton Mediate Nuclear Deformation. In addition to nuclear morphology changes, cell morphology was also altered by surface topography. hMSCs on the flat surface showed normal fibroblast-like spindle shape and formed well-organized cytoskeletal structures; whereas those cultured on micropillars showed elongated cell morphology with a weak assembly of the cytoskeleton (Fig. 1E and Fig. S2A). To investigate the influence of the cytoskeleton on nuclear deformation, hMSCs were treated with 1 μM Latrunculin A, 4 mM acrylamide, and 1 μM colchicine to interfere with the assembly of F-actin, intermediate filament, and microtubule, respectively. After 6 hours of treatment with latrunculin A and acrylamide, a significant increase in NSI was observed, while there was no significant change in NSI after treatment with colchicine indicating the involvement of F-actin and intermediate filaments in nuclear morphology regulation (Fig. S2B, C).²⁰ Additionally, cytoskeleton mediated mechanotransduction processes were also affected by micropillars (Fig. S2D, E).

Micropillars Manipulate 3D Nuclear Architecture and Mechanical Structure. To further determine the influence of micropillars on nuclear architecture, we investigated the 3D structural changes in the cell nucleus. Cell nuclei on flat surfaces exhibited a flatter, 'pancake' shape while nuclei on micropillars were squeezed in-between the pillars with a deformed irregular morphology via contact guidance (Fig. 1F). The projected area of the nucleus on micropillars was significantly smaller, but the height was larger (Fig. S3A, B). According to the 3D reconstruction of nuclei, the nuclear volume and surface area of the deformed nucleus were significantly smaller than the flat condition which

was attributed to the constraints imposed by the micropillars (Fig. 1G, H). Additionally, the ratio of surface area to volume remains unchanged indicating a more compact shape (Fig. S3C).

Nuclear lamins, especially lamin A and C have been reported to play critical roles in regulating nuclear structure and mechanics.²¹ Recent studies revealed that cell culture substrates affect the polymerization of lamin A/C, which in turn influences the polarization of nuclear architecture and epigenetically regulates cell functions.²²⁻²⁴ Lamin A/C staining on flat and micropillar surfaces showed a distinct pattern of nuclear lamins in both horizontal and vertical directions. Horizontally, the lamin A/C was isotropically stained on flat surfaces (Fig. 1I, J and Fig. S3D). However, it was anisotropically distributed in the cell nuclei on micropillars with high intensity of lamins observed at nuclear protrusions and low intensity observed at the nuclear envelope (NE) around micropillars. In addition, distinct lamin A/C wrinkles were observed surrounding micropillars which might be caused by the severe curvature of the cell nucleus at these regions. Vertically, the lamin A/C was anisotropically stained between apical and basal NE in the cell nucleus on a flat surface but was isotropically distributed on micropillars (Fig. 1K). To take into account the influence of absolute intensity variations between samples, the ratios of relative fluorescence intensities between the regions around micropillar and nuclear protrusions, as well as basal versus apical NE were quantified from xy-plane and xz cross-sections, respectively (Fig. 1L, M). This quantification confirmed the distinct distribution pattern of lamin A/C on flat surfaces and micropillars, which might be due to the different cell adhesion geometry and cytoskeleton assembly regulated via contact guidance (Fig. 1N).²² Despite the different distribution patterns, the lamin A/C showed similar expression levels on both flat and micropillar substrates as tested by western blot (WB) analysis (Fig. S3E, F).

Figure 1. Micropillars alter the nuclear architecture and mechanics of hMSCs. **A.** Phase-contrast image of square micropillars with pillar size and space of 5 x 5 μm . **B.** SEM images showing the side and section view of the micropillars. **C.** Typical DAPI staining images of cell nuclei on flat and pillar substrates. **D.** Nuclear shape index of cells on flat (n=273 cells) and pillar substrates (n=295 cells). N=4 experiments. **E.** F-actin and vinculin staining images of cells on flat and pillar substrates. **F.** Representative orthogonal view of cell nuclei on flat and micropillar surfaces showing their 3D structures. **G.** and **H.** are volume and surface area of cell nuclei on flat (n=33 cells) and micropillar (n=34 cells) surfaces. N=3 experiments. **I.** Lamin A/C staining images of cell nuclei on flat and micropillar surface. Solid red arrows indicate strong staining of Lamin A/C; hollow red arrows indicate weak staining of Lamin A/C; yellow arrows indicate wrinkles of Lamin A/C surrounding micropillars. **J.** and **K.** show fluorescence intensity plot of Lamin A/C along selected regions (yellow rectangles 1 and 2 in C) in xy and (yellow rectangles in I) xz plane in the control and deformed nucleus. **L.** and **M.** show the average Lamin A/C intensity ratio at xy and xz plane of cell nuclei on flat (n=25 cells) and micropillar surface (n=26 cells). N=3 experiments. **N.** Schematic summary of influence of micropillars on the polarization of nuclear Lamin A/C and mechanical state. On flat surfaces, actin-caps that formed above the nucleus compress it and the cell adhere to the substrate beneath which leads to burial of the epitope in polymerized lamin A/C at basal NE. On micropillars, the pillar structures prevented the expansion of the nucleus and led to its compression, and provided adhesion with cells that induced multimerization of lamin A/C around micropillars and induced horizontal polarization of NE. (***) $p < 0.001$.

Micropillars Affect Histone Modifications. Although the mechanism remains elusive, growing evidence indicates that biophysical cues can affect the epigenetic state of the cell via the regulation of histone modifications.²⁵⁻²⁷ Therefore, we investigated whether nuclear deformation could lead to alterations in histone acetylation and methylation that are the major types of modifications influencing transcription. After 3 days of culture in maintenance medium, we firstly stained a variety of histone acetylation changes including acetylation of H3 at

lysine 9 (H3K9ac), 14 (H3K14ac), 18 (H3K18ac), and 27 (H3K27ac) (Fig. S4A). Most nuclei showed decreased fluorescence intensity upon nuclear deformation indicating decreased whole-nuclear histone acetylation. A decrease in histone acetylation was further confirmed by detection of global histone H3 acetylation (H3Ac) that was previously reported to affect differentiation of hMSCs (Fig. 2A-C).^{28,29} We then probed for changes in markers of active gene transcription, including methylation of H3 at lysine 4 (H3K4me2) and 36 (H3K36me2 and H3K36me3) and repressive markers including methylation of H3 at lysine 9 (H3K9me3) and 27 (H3K27me3) (Fig. S4B). The majority of the markers remain unchanged between control and deformed nuclei except for H3K27me3, which presented with higher fluorescence intensity and protein expression in cell nuclei on micropillars compared to those on flat surfaces (Fig. 2A-C). Altogether, the global increase in H3K27me3 (repressive) and a decrease in H3Ac (active) in hMSCs cultured on micropillars suggests that such contact guidance may cause transcriptional repression, a response similar to cells undergoing mechanical loading.^{30,31}

To elucidate the upstream pathways that regulated histone modification, we first studied the total protein levels and cellular localization of histone deacetylases (HDAC) including HDAC1, 2, and 3, which have been reported to affect both mechanotransduction and differentiation of hMSCs (Fig. 2D, E and Fig. S4C-E).^{2,27} We identified that the HDAC3 was accumulated into deformed nuclei on micropillars compared to those on the flat surface, although the total cellular levels of HDAC3 remained unchanged. Since enhancer of zeste homolog 2 (EZH2) is currently the only reported histone methyltransferase that catalyzes H3K27me3,³² we then investigated its localization on flat compared to micropillar surfaces (Fig. 2F). According to the staining images, EZH2 is located in both the nucleus and the cytosol on hMSCs cultured on flat surface, but mainly stained in the nucleus of hMSCs on micropillars. A significant increase of EZH2 was also observed on micropillars from WB (Fig. 2G and Fig. 4F). The increased EZH2 we observed in deformed nuclei could thus contribute to the enhanced H3K27me3 repressive histone marker.

Figure 2. Nuclear deformation-induced changes in histone modifications. **A.** Immunostaining images and **B.** western blot images of H3Ac and H3K27me3 in cell nuclei on flat and micropillar surfaces. Total histone H3 and GAPDH are shown as a control. **C.** Relative change of H3Ac and H3K27me3 expression compared to total H3 expression in cells. The relative expression level on flat surface was normalized to be 1 (N=3 experiments). **D.** Immunostaining images of HDAC 3 in cells on flat and micropillar surfaces. White and yellow arrows indicate staining signal in the nucleus and cytosol, respectively. **E.** Intensity ratio of nuclear HDAC3 to cytoplasmic HDAC3 fluorescence intensity per area of cells on flat (n=232 cells) and micropillar (n=238 cells) surfaces. N=3 experiments. **F.** Immunostaining images of EZH2 in cells on flat and micropillar surfaces. White and yellow arrows indicate staining signal in the nucleus and cytosol, respectively. **G.** Relative change of EZH2 expression compared to total H3 expression in cells. The relative expression level on flat surface was normalized to be 1 (N=3 experiments). (* $p<0.05$, * $p<0.05$, *** $p<0.001$).

Nuclear Deformation Causes Significant Downregulation of Processes Associated with Development. Since histone modifications are closely related to transcription profile,³³ we investigated the influence of nuclear deformation on gene expression in hMSCs. We performed RNA-seq analysis on differentially expressed genes, to evaluate the effect of the micropillars on the early transcriptional changes in hMSCs. We observed that the majority of identified differentially expressed genes are downregulated in cells on micropillars when compared to on a flat surface in hMSCs (Fig. 3A, B). Our previous survey of histone modifications indicated a significant increase in H3K27me3 in hMSCs cultured on micropillars. EZH2 catalyzes H3K27me3 and is also a part of the PRC2 complex which is involved in the repression of developmental processes in stem cells.^{32,34} Therefore, we performed gene ontology (GO) analysis on the differentially expressed genes (Table S1) to determine whether similar processes

were associated with the cells cultured on micropillars (Fig. 3C). Since there was more downregulation of genes in hMSCs cultured on micropillars compared to flat surface, we also specifically performed the GO analysis on significantly down-regulated genes (Fig. S5). The enriched processes associated with the identified differentially expressed genes include those associated with cell cycle, DNA conformation, and development. Next, to identify sets of genes with coordinated enrichment or depletion for hMSCs on micropillar compared to flat surfaces, we performed Gene Set Enrichment Analysis (GSEA) for both Canonical Pathways and Gene Ontology Biological Processes gene sets.³⁵ At a False Discovery Rate (FDR) <25%, 273 gene sets were found to be depleted, and 50 gene sets were enriched in micropillars in the Canonical Pathways gene sets. Using the top 20 significantly depleted terms in the pillar conditions, we found significant depletion in gene sets for cell cycle associated pathways (Fig. 3D). Similarly, using the top 20 significantly enriched terms in the pillar conditions, we found that most of the upregulated gene sets were associated with translation and various key signaling pathways involved in MSC differentiation, such as those related to PDGF, interleukins, and Rho GTPases (Fig. 3E). Furthermore, we noticed enrichment in gene set associated with chromatin-modifying enzymes (related to histone acetyltransferases, histone deacetylases, histone lysine methyltransferases, and histone lysine demethylase, Fig. S6). This finding confirmed the influence of micropillars on the epigenome as a consequence of nuclear deformation. Similarly, we also identified the top 20 significantly enriched and depleted Gene Ontology gene sets for both flat and pillar cell culture conditions which were consistent with results from Canonical Pathways gene sets (Table S3). Altogether, our results show that nuclear deformation impacts the transcriptional profile in hMSCs to alter developmental processes such as differentiation and proliferation that are inherently linked with each other.

Nuclear Deformation Alters Chromatin Conformation within Packing Domains in hMSCs. Chromatin packing scaling is a key physical property that's tightly associated with transcription regulation and is also a crucial regulator of phenotypic plasticity.¹⁷ To directly investigate the influence of nuclear deformation on chromatin reprogramming, we measured chromatin packing scaling from the level of packing domains in the mechanically constrained nucleus using partial-wave spectroscopic (PWS) microscopy that is capable of measuring chromatin packing scaling with sensitivity to length scales as small as 20 nm. Additionally, PWS enables the label-free sensing of nanoscale variations in supranucleosomal chromatin structure in both living and fixed cells.³⁶ Specifically, the variations in the chromatin packing density are measured using PWS in the form of a spectral interference signal originating from internal scattering within the cell nucleus. The shape of the autocorrelation function (ACF) of the chromatin density variations or

Figure. 3. Micropillars cause downregulation of developmental processes in hMSCs. **A.** Volcano plot displaying differentially expressed genes in hMSCs seeded on micropillars compared to the flat surface. A negative fold change indicates genes downregulated in the micropillar condition compared to flat. **B.** Top 100 Differentially Expressed genes in cells seeded on the flat surface compared to control based on absolute log fold change in hMSCs. **C.** Gene Ontology (GO) analysis for the top 20 most enriched processes in cells seeded on micropillars compared to a flat surface in hMSCs. (List of genes annotated to each process can be found in Table S2) **A** network analysis of the top 20 canonical pathways gene sets **D.** depleted, and **E.** enriched in pillars found using GSEA. Processes and pathways in **D** and **E** are colored by cluster-ID, where nodes that share the same cluster-ID are typically close to each other (terms with a similarity, Kappa Scores > 0.3 are connected by edges).

interference signal is then evaluated to determine the average nuclear chromatin packing scaling, D .^{18,37} As shown in Fig. 4A-C, hMSCs seeded on micropillar surface compared to those on the flat surface showed a decrease in

whole-nuclear chromatin packing scaling of about $8.01 \pm 0.74\%$ (SEM) in maintenance medium within 24 hours. Compared to the effect of other external cues, such as treatment with various pharmacological agents for similar durations, such a change in D using morphological cues is indicative of a drastic change in chromatin conformation.^{17,38} In addition, we investigated how micropillar induced changes in chromatin packing scaling compared with those during osteogenic differentiation of hMSCs. We observed a significant decrease in chromatin packing scaling after Day 1 of osteogenic induction. Such a decrease in chromatin packing scaling was also maintained in osteogenic differentiating cells at Day 4 and Day 14 of induction (Fig. S7). Chromatin packing scaling of hMSCs was higher compared to osteogenically induced progenitor and differentiated cells, consistent with our previous observations that higher D is associated with phenotypic plasticity.¹⁷ Furthermore, by decreasing D , micropillars, may offer an efficient way to modulate this plasticity associated with hMSCs in order to enhance differentiation efficiency towards a target lineage.

In order to elucidate the effects of nuclear deformation on chromatin conformation below 20 nm, we employed ChromTEM to image chromatin in uninduced hMSCs grown on micropillars versus flat surfaces (Fig. 4D). Since the nuclear deformation resulted in a significant impact on the lamin A/C organization, we hypothesized that the lamina-associated heterochromatin would be altered in micropillars. Previous electron microscopy studies have revealed that the nuclear periphery is enriched in condensed heterochromatin.³⁹ Additionally, the nuclear envelope and chromatin organized into compacted domains may contribute to the response of the cell nucleus to mechanical forces.⁴⁰ Firstly, we observed that the mass density ratio of the peripheral chromatin to non-peripheral chromatin significantly decreases in the deformed nucleus compared to the flat surface (Fig. S8). Therefore, we specifically segmented the peripheral chromatin region and analyzed changes in D within this region. We then evaluated the average ACF of chromatin mass density for both the whole nucleus, and peripheral chromatin of hMSCs on micropillars compared to flat surface within 50-200 nm (Fig. 4E). Next, to determine chromatin packing scaling of these different regions, we fit a linear regression to the chromatin density ACF within the whole nucleus, and the peripheral domains for each cell from both groups (hMSCs on flat and micropillars). We measured a $10.82 \pm 3.1\%$ (SEM) decrease in chromatin packing scaling for the whole nucleus in micropillars compared to flat surface was measured, which was comparable to the changes in nuclear D obtained using PWS. In addition, we observed an $11.02 \pm 2.2\%$ (SEM) increase in chromatin packing scaling for peripheral chromatin in micropillars compared to flat surface was obtained (Fig. 4F). These findings indicate that upon nuclear deformation the anchoring of the chromatin to the periphery of the nucleus may be altered resulting in an increased heterogeneity in the peripheral chromatin domains. Additionally, this increased heterogeneity in the peripheral chromatin might be a consequence of the anisotropic distribution of lamin A/C on micropillars.⁴¹

Figure 4. Chromatin Packing Scaling Decreases in Deformed hMSC Nuclei. PWS microscopy of hMSCs and osteogenic differentiating hMSCs show a significant change in average chromatin packing scaling, D in deformed nuclei compared to control. Stem cells on **A.** flat and **B.** micropillar surfaces show changes in packing domains (red clusters). **C.** Micropillars deform cell nuclei and significantly decrease average D in stem cells for $n=111$ hMSCs on flat surface and $n=110$ hMSCs on pillar surface. $N=4$ experiments. **D.** ChromTEM images of 50 nm thick resin sections of hMSCs seeded on flat and micropillar surface. The technique resolves nucleoli, nuclear speckles, and mitochondria at high resolution. Orange arrows show the location of micropillars. **E.** Spatial autocorrelation function (ACF) of chromatin density in the log-log scale for the whole nucleus, and peripheral chromatin. We obtained the chromatin packing scaling by performing a linear regression of the ACF in the log-log scale within the ranges of 80-200 nm for the whole nucleus and 50-75 nm for peripheral chromatin. **F.** Chromatin packing scaling shows the significant difference for whole-cell nuclei and periphery of nuclei cultured on flat ($n=20$ hMSCs) and

pillar surfaces (n=12 hMSCs), indicating a drastic change in the chromatin organization. (** $p<0.01$, **** $p<0.0001$). N=2 experiments.

Nuclear Deformation Increases Transcriptional Responsiveness of hMSCs to Osteogenic Induction with enhanced Wnt/ β -catenin signaling.

If transcriptional plasticity of stem cells is altered, this could enhance their ability to respond to a given induction cue and in turn, increase their differentiation efficiency towards a specific lineage.¹⁷ Therefore, we evaluated the lineage-specific responsiveness coefficient, R_{LS} defined as the average transcriptional response to an external differentiation stimulus of stem cells on a micropillar surface compared to a flat surface,

$$R_{LS} = \frac{\frac{E_{Induced,Pillar}}{E_{Stem,Pillar}}}{\frac{E_{Induced,Flat}}{E_{Stem,Flat}}} \quad (1)$$

Here E denotes the expression rate of a specific gene, subscripts “induced” and “stem” refer to osteogenic differentiation induction and control conditions of stem cells, respectively. Using our bulk RNA-Seq data, genes were grouped based on initial pre-stimulated expression and their change in average expression in response to an osteogenic differentiation stimulus was quantified in flat and pillar populations (Fig. 5A). An increase in R_{LS} during hMSC differentiation in our case would indicate an increase in lineage-specific-transcriptional response in micropillar compared to a flat surface. First, we employed our previously developed CPMC model, inputting our experimentally determined chromatin packing scaling for the micropillar versus flat surfaces, to predict the lineage responsiveness coefficient for genes that are upregulated and downregulated by differentiation induction. For initially lowly expressed genes that are downregulated in the stem cell state, there is further downregulation in the lower D cells on micropillars compared to the higher D stem cells on the flat surface, which is shown by an increase in R_{LS} upon stimulation with osteogenic differentiation cues (Fig. 5B, C, orange curve). A similar trend was determined for the initially highly expressed genes, although the magnitude of the change was much smaller (Fig. 5B, C, purple curve). Altogether, the model predicted that, for lower D cells compared to higher D cells, genes with higher initial expression in stem cell condition did not show as much downregulation of genes associated with the stem state as the genes with initial lower expression. Next, we checked if such changes are also observed experimentally by analyzing our bulk RNA-seq data. In agreement with our model predictions, we observed that the lineage-specific transcriptional response to osteogenic differentiation induction increased in low D cells on pillars compared to high D cells cultured on flat surfaces in response to differentiation induction as R_{LS} was >1 for the majority of the group of genes grouped by initial control expression (Fig. 5B, C). Notably, genes with initially low expression in the control stem cell population exhibited a greater change in their global transcriptional profile on induction compared to initially highly expressed genes as predicted by the model. Additionally, we also observed increased downregulation compared to upregulation of genes in induced cells compared to stem cells on Day 1 after differentiation. These genes associated with the stem cell state had stronger further downregulation in pillars compared to a flat surface upon differentiation induction as predicted by the model. Furthermore, the genes associated with differentiation were similarly upregulated in low D cells on pillar versus high D cells on flat surfaces. Therefore, the ability of hMSCs cultured on micropillars to differentiate more efficiently is due to increased downregulation of stem cell-associated genes instead of their ability to increase the expression of genes associated with differentiation.

To identify the transcriptional processes that guide changes in transcriptional responsiveness in cells on micropillars, differential gene expression combined with gene ontology analysis was employed to determine the

upregulated and downregulated processes in induced cells compared to control cells on flat surfaces. The processes identified in Fig. 5D demonstrate a large cluster annotated to development-specific processes such as urogenital system development, blood vessel morphogenesis, epithelial cell differentiation, muscle structure development, etc.. Of these, there is an even smaller cluster of bone development processes which included ossification, connective tissue, and skeletal system development. Additionally, we analyzed the effect of genes in these processes by evaluating the lineage-specific responsiveness coefficient, R_{LS} , for stem cells on micropillars compared to flat surfaces (Fig. 5E). We notice that these development-specific genes follow a similar trend as previously observed, although less drastic as the identified differentially expressed genes in Fig. 5B, C. Altogether, these results indicate that micropillars increase the overall response of lineage-specific genes with initial low expression in differentiating hMSCs, which may contribute to their increased osteogenic differentiation efficiency.

Several potential signal transduction pathways regulate osteogenic differentiation of hMSCs upon induction such as Wnt, BMP, Hedgehog, Notch signaling.⁴² Based on our RNA-seq results, we noticed that Dickkopf-related protein 1 (DKK1), an inhibitor of canonical Wnt signaling, was the most significantly altered gene between hMSCs on flat and micropillar surface after induction (Fig. 5F and Fig. S9). Interestingly, several Wnt signaling-related genes including Dishevelled (DVL3, DVL1, wnt conductor), and Transducin-like enhancer protein 4 (TLE4, Wnt co-repressor) were also found to be significantly altered in cells seeded on micropillars compared to flat surfaces. Therefore, we hypothesized that Wnt signaling might be promoted on micropillars as the responsiveness of hMSCs to osteogenic induction increases. To test this hypothesis, we stained β -catenin and RUNX2 on the same substrate containing both flat and micropillar surfaces (Fig. 5G and Fig. S10A). β -catenin and RUNX2 showed obvious nuclear accumulation on both surfaces. However, the stronger fluorescence intensity of both β -catenin and RUNX2 was observed on micropillars. Quantification of absolute nuclear intensity and relative intensity ratio of nuclear/cytoplasmic confirmed the enhanced nuclear accumulation of β -catenin and RUNX2 on micropillars (Fig. 5H, I and Fig. S10B, C). These results suggest that nuclear deformation on micropillars promotes the activation of canonical Wnt signaling upon induction to facilitate the osteogenic differentiation of hMSCs.

To investigate the effects of nuclear deformation on cellular phenotype, we then evaluated the influence of micropillars on osteogenic differentiation of hMSCs upon induction. Alkaline phosphatase (ALP) quantification demonstrated that micropillars promoted osteogenic differentiation of hMSCs (Fig. 5J). Both early (ALP) and late (integrin-binding sialoprotein, IBSP) osteogenic related genes showed increased expression on micropillars (Fig. 5K). Additionally, calcium deposition in cells was also enhanced on micropillars (Fig. S10D, E). Interestingly, the differentiated hMSCs maintained and even further decreased NSI which may be due to enhanced cytoskeletal tension after osteogenic differentiation (Fig. S10F, G).⁴³ These results are in accordance with a previous study that reported an increase of osteogenic differentiation of mouse hMSCs on micropillars.¹³

Figure 5. Micropillars Increase Lineage Specific Response by decreasing D in hMSCs. **A.** Mesenchymal stem cells seeded on a flat surface (high D) and micropillar surface (low D) (SF and SP, respectively) were induced to differentiate towards osteogenic lineage (IF and IP, respectively). E denotes expression rate in the respective conditions used to evaluate lineage-specific responsiveness coefficient, R_{LS} , a measure of response to differentiation induction (osteogenic differentiation in this case) on the pillar surface compared to flat surface. **B.** Lineage-specific transcriptional response due to differentiation induction increases in low D cells for 3323 differentially expressed genes with p -adjusted<0.05 and **C.** 1513 genes with p <0.05 and |fold change|>1.5 for high (orange) and low initial expressions (purple) in the control cells (SF). Here, each dot represents 126 genes (in B) and 57 genes (in C). CPMC Model predictions for initially lowly expressed genes and initially highly expressed

genes are shown in orange and purple respectively. **D.** A network of top 20 pathways and processes identified from all DE genes ($p\text{-adj}<0.05$ and $|FC|>1.5$) in induced cells compared to stem cells on a flat surface: colored by cluster-ID, where nodes that share the same cluster-ID are typically close to each other (terms with a similarity, Kappa Scores > 0.3 are connected by edges). **E.** Lineage-specific transcriptional response due to differentiation induction increases in low *D* cells for all DE genes with $p\text{-adj}<0.05$ and $|FC|>1.5$ (where each blue dot represents 150 genes) and genes in development-specific processes (highlighted cluster in green) where each green dot represents 57 genes, and bone development genes (highlighted in red) where each dot represents 12 genes. (List of genes annotated to each process can be found in Table S4) **F.** Log2 Fold Change in gene expression of DKK1 (Wnt inhibitor), DVL3, and DVL1 (Wnt conductor), as well as TLE4 (Wnt co-repressor) on flat and micropillar substrates upon osteogenic induction. A negative fold change indicates downregulation in micropillars compared to the flat surface. **G.** Representative immunofluorescent images of β -catenin and RUNX2 in cells. **H.** and **I.** show the intensity ratio of nuclear/cytoplasmic β -catenin and RUNX2 in cells. $n=187$ and 199 cells on flat and micropillar surfaces, respectively. $N=3$ experiments. **J.** ALP staining images and ALP activity analysis of cells after 7-day osteogenic differentiation induction ($N=4$ experiments). **K.** Relative gene expression of ALP and IBSP tested after osteogenic induction for 7 days of cells on flat and micropillar substrates ($N=3$ experiments). ($*p<0.05$, $***p<0.001$).

Discussion

This work demonstrates that contact guidance-induced nuclear morphology changes modulated histone modifications and reprogrammed chromatin, which in turn influenced cell plasticity and transcriptional responsiveness of stem cells to external cues. These effects ultimately modulate cell phenotype as summarized in Fig. 6. Deformed nuclei showed a 3D configuration similar to nuclei cultured in 3D scaffolds, which had a more folded structure and favored osteogenesis despite weak cytoskeleton assembly.⁴⁴ The nuclear deformation affected nuclear lamin A/C multimerization which regulates the mechanical properties and mechano-sensing and -transduction of the nucleus.^{21,45} Since nuclear lamins provide anchoring sites for multiple chromatin domains, alteration in their polarization pattern may also affect the chromatin organization and transcription.⁴¹ In response to micropillar topography, cells assembled a weak cytoskeleton, which resulted in the shuttling of HDAC3 to the nucleus attributed to the release of HDAC3 from the I κ B complex that dissociated from less assembled F-actin filaments on micropillars.⁴⁶ Additionally, previous studies reported the existence of cytosolic EZH2 that form methyltransferase complex in association with Vav1 and Talin1, which regulate cell adhesion and actin polymerization.^{47,48} Therefore, the strong F-actin assembly and FAs formation on flat surfaces may facilitate the shuttling of EZH2 to the cytosol. Changes in nuclear localization of these histone-modification enzymes significantly affected cellular epigenetics with a decrease in activating histone acetylation and an increase in repressive histone methylation in the deformed nucleus. Correspondingly, there was a decreased transcriptional activity of the majority of development-related genes. These results suggest a decreased cell plasticity in the deformed nucleus which may prevent off-targeted differentiation of hMSCs.⁴⁹ Further studies are needed to determine if histone modifications drive these transcriptional changes or if the histone modifications themselves are a downstream product of transcriptional change.

Chromatin reprogramming in deformed nuclei was directly observed using PWS microscopy and ChromTEM. The results confirmed that compared to a flat surface, pillars cause a significant decrease in *D* in the whole nuclei. Our previous study of structural and temporal changes of chromatin architecture using dual-PWS has shown that hMSCs have increased variations in both chromatin packing density and macromolecular motion within the nucleus than osteoblasts derived from them.⁵⁰ Based on the previously established CPMC model of transcription,

D is directly related to transcriptional responsiveness. Furthermore, using cancer as a testbed, it has been demonstrated that chromatin packing scaling increases the phenotypic plasticity of cancer cells by determining the responsiveness of the cells to external cues. Cancer cells with higher D are more likely to survive cytotoxic stressors because of their ability to both upregulate survival genes within a critical time period (i.e. their transcriptional malleability) and their inherent transcriptional heterogeneity which increases the spread in available transcriptional states that a cell population can sample from.¹⁷ In accordance with CPMC model predictions, RNA-seq analysis combined with results from our microscopy experiments showed a decrease in D in hMSCs nuclei using pillars is accompanied by an increase in the stem cells' ability to respond to osteogenic differentiation induction at an early time point which is in accordance with the CPMC model. The decrease in D of stem cells cultured on micropillar surfaces resulted in decreased expression of downregulated genes which are downregulated upon differentiation induction. Both our model predictions and experimental results demonstrate that differentiation on micropillars is more efficient than differentiation on flat surfaces, as micropillars can decrease chromatin packing in hMSCs and consequently increase downregulation of genes associated with the stem cell state. Therefore, contact guidance-induced chromatin reprogramming is a powerful tool that can be used to ultimately increase the differentiation efficiency of stem cells.

Figure 6. Schematic illustration of the effects of micropillars on osteogenic differentiation of hMSCs. Compared to that on a flat surface, cell nuclei on micropillars had an anisotropic distribution of lamin A/C. A decrease in chromatin packing scaling in the whole nucleus was observed using PWS and ChromTEM. Epigenetic changes like an increase in repressive histone modifications like H3K27me3 and a decrease in activating modifications H3 acetylation were observed on micropillars. Such epigenomic changes may lead to downregulation of the majority of genes on micropillars. As a consequence of the decrease in chromatin packing scaling, lineage-specific responsiveness upon osteogenic induction was increased in micropillars.

Herein, we demonstrate enhanced osteogenic differentiation of hMSCs due to contact guidance-induced chromatin reprogramming on micropillars fabricated on mPOC, a citrate-based biomaterial (CBB). CBBs have been shown to regenerate bone and are compatible with microfabrication techniques, are biodegradable, and elicit non-toxic, anti-inflammatory responses.^{51,52} CITRELOCK™, a biodegradable orthopedic fixation device fabricated from a CBB was recently approved by the U.S. Food and Drug Administration (FDA) ([K200725.pdf \(fda.gov\)](#)). Engineering the topography of orthopedic devices to include micropillars may result in enhanced bone apposition, improving device function and patient outcomes. Overall, our findings highlight how manipulating nuclear morphology using topographically engineered surfaces impacts chromatin reprogramming and gene transcription to control cell functions, which can in the future be used for various applications such as bone regeneration.

Although our results are promising, further studies are required to determine the role of D in facilitating differentiation. Specifically, it remains to be explored how nuclear reprogramming, influences the movement of genes between and within domains in the 3D genome, thus altering their expression patterns.⁴⁶ Studies involving chromatin conformation capture methods, gene labeling, combined with sequencing can be synergistically used to explore how a change in the spatial location of groups of genes, such as those associated with lineage/development are able to modulate their expression and eventually determine the cell fate or phenotype as a result of nuclear deformation.

Materials And Methods

Synthesis and Characterization of mPOC Pre-polymer. POC pre-polymer was firstly prepared according to the previous report.⁵³ Briefly, equal molar of citric acid and 1,8-octandiol were melted at 160 °C. Then, the mixture was transferred into a 140 °C oil bath and reacted for 30 min. The mixture was cooled down and dissolved in ethanol and purified by precipitation in DI water. The pre-polymer was lyophilized and used for the methacrylation process. 66 g POC pre-polymer was dissolved in 540 ml tetrahydrofuran (THF) and placed in a 60 °C water bath with stirring. Next, 0.036 mol imidazole was added into the system followed by drop-wise adding 0.4 mol glycidyl methacrylate. After reacting for 6 h, the solvent was removed using a rotary evaporator. The remaining product was purified by precipitation in DI water and lyophilized for further application. 5 mg mPOC pre-polymer was dissolved in 1 ml deuterated dimethyl sulfoxide (DMSO-d₆) and characterized using proton nuclear magnetic resonance (¹H-NMR).

Fabrication and Characterization of Micropillar Substrates. The mPOC micropillars were fabricated using a combination of contact printing and UV lithography. Several features including micropillar size, shape, spacing, and height were designed to regulate cell nuclear deformation. Hard micropatterned master molds were firstly fabricated using photolithography. Briefly, a 4 inches Si wafer was pre-treated using a reactive ion etcher (RIE-10NR, SAMCO Inc.) followed by a spinning coating of SU-8 photoresist (SU8 3010) at a 4000 rpm for 30s. The photoresist was then soft baked at 65 °C (3 min) and 95 °C (2 min), respectively. The micro-features were transferred to the photoresist using the MLA150 mask-less aligner. After UV exposure, the photoresist was post-baked at 65 °C (1 min) and 95 °C (2min), and developed in SU-8 developer followed by a rinse with isopropanol and DI water. Hard bake at 150 °C for 10 min was performed to ensure the photoresist strength for repeated use. A PDMS mold replicating the micro-features was made by curing PDMS precursor (120 °C, 30 min) on the master mold. Finally, 50 µl mPOC pre-polymer (70% v/v in ethanol) mixed with photo-initiator (5 mg/ml camphorquinone and ethyl 4-dimethylaminobenzoate) was added onto the PDMS (2 x 2 cm²) mold and covered with an oxygen plasma pre-treated cover glass. The polymer was cured by exposure to 405 nm laser light with a power of 1W for 2 min. After peeling off the PDMS mold, the mPOC micropillars were fabricated onto the cover glass. The prepared microfeatures were characterized by SEM (FEI Quanta 650 ESEM) and 3D Optical Microscope (Bruker). The mPOC micropatterns were sterilized using ethylene oxide and kept in DPBS before cell culture. Flat mPOC films were fabricated using the same method as flat PDMS molds. To fabricate substrates for ChromEM imaging, micropillars were directly fabricated on a cover glass using SU8-3010 which can reduce background and facilitate sample sectioning.

Cell Nuclear Deformation on Micropillars. Human mesenchymal stem cells (hMSCs, PCS-500-012) were purchased from the American Type Culture Collection (ATCC) and sub-cultured using a growth medium acquired from the same company. hMSCs (P4-P6) were seeded onto the flat mPOC substrates and the mPOC micropatterns with various microfeatures. After one-day culture, the cells were fixed with 4% paraformaldehyde, and cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) to show nuclear morphology. Nuclear shape index (NSI) was calculated according to the staining images using the following equation: $NSI = 4\pi A/p^2$, in which A represents the area and p represents the perimeter. A total of 273 nuclei on flat surfaces and 295 nuclei on micropillar surfaces from 4 biological replicates were imaged and analyzed to calculate the statistics. In order to acquire 3D nuclear morphology, the stained cells were imaged using a confocal microscope (Leica SP8). The acquired images were analyzed using Fiji ImageJ software (<https://imagej.net/Fiji>) to measure cell nuclear volume, surface area, project area, height, and the ratio of surface area to volume (3D objects counter). A total of 33 nuclei on flat surfaces and 34 nuclei on micropillar surfaces from 3 biological replicates were imaged and analyzed to calculate the statistics.

Cytoskeleton Inhibition. hMSCs were cultured on micropillars for one day before treatment of pharmacological agents. Specifically, F-actin filaments, microtubules, and intermediate filaments were disrupted with 1 μ M Latrunculin A, 1 μ M colchicine; 4mM acrylamide, respectively. 6 h post-treatment, drugs were washed out and the cells were fixed and stained with Hoechst to show the nuclear morphology.

Immunostaining, Imaging, and Quantification. Cells on flat and micropillar surfaces were fixed and permeabilized with 0.1% Triton-X100 followed by blocking with 1% BSA solution. Then, the primary antibody (detail in Supplementary Table S6 and S7) were diluted in blocking solution and incubated with cells at 4°C overnight. After washing with PBS buffer, secondary antibodies and Hoechst were diluted 1:1000 in PBS and incubated with cells at room temperature for 1h. The fluorescent images were acquired using a cytation 5 imaging reader and a Nikon eclipse TE2000-U inverted microscope. Histology images were analyzed using ImageJ (1.50 i, NIH, [Download \(nih.gov\)](https://download.nih.gov/pub/nihs/Download.do)) according to a previous report.^{54,55} The nuclear YAP positive cells were defined as those that had a nuclear/cytoplasmic intensity ratio larger than 2. Lamin A/C staining images were acquired using a confocal microscope and the intensity plot was analyzed using ImageJ. A total of 25 and 26 nuclei on flat and micropillar surfaces, respectively, from 3 biological replicates were imaged and analyzed to calculate the statistics. Histone acetylation and methylation markers were imaged to qualitatively evaluate the influence of nuclear deformation on cell epigenetics. HDAC 1, 2 and 3, and EZH2 were stained to study the upstream effects of histone modifications. A total of 232 and 238 cells on flat and micropillar surfaces from 3 biological replicates, respectively, were imaged and analyzed to calculate the statistics. β -catenin and RUNX2 were probed to study the effects of nuclear deformation on canonical Wnt signaling. A total of 187 and 199 cells on flat and micropillar surfaces from 3 biological replicates, respectively, were imaged and analyzed to calculate the statistics. To investigate nuclear shuttling of HDAC3, β -catenin and RUNX2, their nuclear and cytoplasmic fluorescence intensity was analyzed according to the staining images. Briefly, a rectangular frame was created in cell nucleus. The overlap region was selected as region of interest (ROI). The mean fluorescence intensity (M_N) of ROI was measured. Then, the frame was randomly set in the cytoplasm to detect the mean intensity of plasm (M_P). Subsequently, the mean intensity (M_B) of the unoccupied region (no cell region) was measured as the background. Therefore, the nucleus to cytoplasm intensity ratio (R) can be described as: $R=(M_N-M_B)/(M_P-M_B)$.

Western Blot Analysis. hMSCs on different substrates were lysed using radioimmunoprecipitation assay (RIPA) buffer containing 150 mM NaCl, 0.1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS), 50 mM Tris-HCl and 1% protease inhibitors. Protein lysates were then centrifuged to remove cell debris and any other undissolved component. The relative quantity of proteins was measured using cytation 5 imaging reader. Equal amounts of proteins from flat and micropillar samples were run using a NuPAGE 4-12% Bis-Tris Gel (Invitrogen) and transferred to nitrocellulose membranes (Bio-rad). The membranes were then blocked using 5% milk and incubated with primary antibodies (including GAPDH, lamin A/C, total H3, H3Ac, H3K27me3, HDAC1, 2, and 3, and EZH2, Supplementary Table S6 and S7) at 4 °C with gentle shaking overnight. Secondary antibodies were diluted at a ratio of 1:5000 and incubated with the membranes at room temperature for 1h. Protein bands were visualized using an ImageQuant LAS 4010 Gel Imager (Cytiva). The acquired images were analyzed using 'Gel Analyzer' in ImageJ. The intensity of all target protein band was firstly compared with relevant total H3 and then normalized with flat surface that was set as 1. Three biological replicates were used to calculate statistics.

PWS Microscopy. Bone Marrow-Derived Mesenchymal Stem Cells (hMSCs) were cultured in 35 mm glass-bottom Petri dishes (Cellvis, Mountain View, CA) with a micropatterned or flat surface in growth medium or osteogenic differentiation medium at 37°C and 5% CO₂.

The PWS microscopy images were acquired on a commercial inverted microscope (Leica, Buffalo Grove, IL, DMIRB) with a Hamamatsu Image EM charge-coupled device camera (C9100-13) coupled to a liquid crystal tunable filter (CRi, Woburn, MA) to collect spectrally resolved images between 500 to 700 nm with 1 nm step size. Further, broadband illumination is provided by an Xcite-120 LED lamp (Excelitas, Waltham, MA). PWS microscopy was used to capture spatial variations of the refractive index distribution or chromatin packing density heterogeneity (Σ) within the nucleus. Further, the statistical parameter of chromatin structure, packing scaling (D) was calculated from Σ .³⁷ At least 10 independent fields of view were utilized for each experiment and four biological replicates were used for the analysis. D value was calculated for 111 hMSCs from the flat surface, and 110 hMSCs on pillar surfaces.

ChromTEM Sample Preparation, Image Acquisition, and Analysis. ChromTEM staining targets nuclear DNA specifically by utilizing the “click-EM” method.⁵⁶ Compared to conventional negative staining, which ubiquitously labels nucleic acid by uranyl acetate and lead citrate, ChromTEM provides us an opportunity to investigate chromatin organization from the perspective of DNA packing at high resolution. The image contrast for ChromTEM at bright-field for a thin resin section follows Beer’s Law, which can be converted to DNA concentration with calibration:

$$I(x, y) = I_0 e^{-\sigma \rho(x, y) t} \quad (1)$$

Here $I(x, y)$ is the intensity of the resultant image, I_0 is the intensity of the incident beam, σ is the absorption coefficient of the sample, $\rho(x, y)$ is the density distribution, and t is the thickness of the section consisting of the sample. We assumed that for a given resolution, the absorption coefficient is constant. Further incident beam and section thickness was controlled to be the same across all images. Therefore, after taking a negative logarithm of the image followed by subtraction of the mean from the image, we directly obtain the chromatin density fluctuations from the image intensity.

The chromatin density fluctuations can then be used to estimate *ACF* (autocorrelation function of chromatin density) using the Weiner Kinchen relation as previously described.⁵⁷ D can be evaluated using the power-law relationship of *ACF* approximated by:

$$ACF \sim r^{D-3} \quad (2)$$

where r is the spatial separation. This is followed by linear regression analysis to obtain the chromatin packing scaling for a given region within the nucleus. We were able to evaluate local chromatin packing D at different length scales by linear regressions on *ACF* in log-log scale.

Cells were prepared for TEM imaging using the ChromEM staining protocol⁴². hMSCs seeded on a micropatterned or flat surface in growth medium were washed with Hank’s balanced salt solution without calcium and magnesium. Next, hMSCs were fixed for 5 minutes at room temperature with EM grade 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1M sodium cacodylate buffer (EMS) and the fixation was continued for 1 hour on ice after refreshing the samples with fresh fixative, and all consecutive steps were carried out in the ice. Cells were washed with 0.1M sodium cacodylate buffer five times for 2 minutes each, blocked with potassium cyanide

(Sigma Aldrich) blocking buffer for 15 minutes, and stained with DRAQ5[™] (Thermo Fisher) with 0.1% saponin (Sigma Aldrich) for 10 minutes. Then the samples were washed with blocking buffer. Samples were then moved to a pre-cooled custom-made chamber. Photobleaching was done on samples in the chamber bathed in 3-3' diaminobenzidine tetrahydrochloride (DAB) solution (Sigma Aldrich) and using a Nikon inverted microscope (Eclipse Ti-U with the perfect-focus system, Nikon) with Cy5 filter set, along with a 15 W Xenon lamp and the red filter for epi-illumination. Total photobleaching time per sample was about 20 minutes using a 20X objective. Once photobleached, the samples were washed with 0.1 M sodium cacodylate buffer, about five times for 2 minutes each, and the samples were then stained with reduced osmium (2% osmium tetroxide and 1.5% potassium ferrocyanide, EMS) for 30 minutes. Further, the cells were washed with DI water. This was followed by serial ethanol dehydration at 30%, 50%, 70, 85%, 90%, and 100% (x2), with the final 100% ethanol added at room temperature, unlike the preceding steps. Infiltration and embedding using Durcupan resin (EMS) were performed and the samples were then cured at 60°C for 48 hours. 50 nm thick resin sections were prepared using an ultramicrotome (UC7, Leica). TEM (HT7700, HITACHI) was operated at 80 kV in a bright field to acquire high contrast images of the samples. For image analysis, the nuclei were segmented using the Image Segmenter toolbox on MATLAB. Further, peripheral chromatin was segmented by creating a mask of 150 nm (median evaluated from a total of 716 data points of peripheral heterochromatin thickness from 11 cells) in thickness from the nuclear periphery. The ACF was calculated from all the cells in both flat and pillar groups for the whole nucleus, and the peripheral chromatin to evaluate the D value by log-log fitting from $r = 80$ nm to $r = 200$ nm and from $r = 50$ nm to 75 nm, respectively. For statistical analysis, the D value calculated for 20 hMSC on flat surface, and 12 hMSCs on micropillar surface. Imaging was performed on four independent 50 nm sections from two biological replicates from each group (hMSCs on flat and hMSCs on micropillar surfaces).

Differential Gene Expression and Gene Ontology analysis. RNA extraction was performed on samples from flat and micropillar surfaces in both regular and osteogenic differentiation medium with two biological replicates per condition. Sequencing and library preparation was performed by Northwestern University NUSC Core Facility. Illumina HiSeq 4000 Sequencer was used to sequence the libraries with the production of single-end, 50–base pair reads. The quality of reads, in fastq format, was evaluated using FastQC (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Adapters were trimmed, and reads of poor quality or aligning to rRNA sequences were filtered using Trim Galore (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). The cleaned reads were aligned to the human genome (hg19) using STAR.⁵⁸ Read counts for each gene were calculated using HTSeq-Counts⁵⁹ in conjunction with a gene annotation file for hg19 obtained from Ensembl (<http://useast.ensembl.org/index.html>). A comprehensive QC report was generated using MultiQC.⁶⁰ Differential expression was determined using DESeq2.⁶¹ The cutoff for determining significantly differentially expressed genes was an FDR-adjusted p-value less than 0.05. The pathway analysis was done using Metascape.⁶²

Osteogenic Differentiation of hMSCs. hMSCs were seeded onto both flat and micropillar substrates. One-day post-seeding, an osteogenic induction medium (Lonza) was used to induce the osteogenic differentiation of hMSCs. After 7 d induction, the cells were washed with PBS buffer followed by fixation with 4% paraformaldehyde for 10 min. Immediately, the samples were merged in a solution of 56 mM 2-amino-2-methyl-1,3-propanediol (AMP, pH~9.9) containing 0.1% naphthol AS-MX phosphate and 0.1% fast blue RR salt to stain ALP. The bright field images were then acquired using a Nikon Eclipse TE2000-U inverted microscope. The ALP activity was tested using the ALP assay kit (K422-500, Biovision) followed by the manual. Briefly, cells that have been cultured in induction medium for 7 d were homogenized using ALP assay buffer. Then, the non-fluorescent substrate 4-

Methylumelliferyl phosphate disodium salt (MUP) was mixed with the homogenized samples to generate a fluorescent signal due to its cleavage by ALP. The fluorescence intensity was read by a cytation 5 imaging reader (BioTek) at (Ex/Em = 360/440 nm). The enzymatic activity was calculated based on the standard curve and normalized to total DNA content tested by the Quant-iT PicoGreen dsDNA assay (Invitrogen). Four biological replicates were used to calculate statistics. The expression of ALP and IBSP of hMSCs on flat and micropillar substrates was evaluated using quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR) after 7d induction. The total RNA of the cells was extracted using the Aurum Total RNA Mini Kit (bio-rad) according to the protocol. The concentration and purity of the extracted RNA were tested using the citation 5 imaging reader. The RT-qPCR was carried out using iTaq Universal Sybr Green One-step Kit following the vendor's protocol. The designed primers for ALP are: *Forward*, 5'-GACCCTTGACCCCCACAAT-3'; *Reverse*, 5'-GCTCGTACTGCATGTCCCCT-3'. The designed primers for IBSP are: *Forward*, 5'-TGCCTTGAGCCTGCTTCC-3'; *Reverse*, 5'-GCAAAATTAAAGCAGTCTTCATTTTG-3'. We employed GAPDH as the house-keeping gene. The GAPDH primers are: *Forward*, 5'-GTGGACCTGACCTGCCGTCT-3'; *Reverse*, 5'-GGAGGAGTGGGTGTCGCTGT-3'. The data was analyzed using the $2^{-\Delta\Delta C_t}$ method. The expression of target genes was firstly normalized to that of GAPDH, and then to the average values on flat substrates. Three biological replicates were used to calculate statistics. Calcium deposition were stained with Alizarin Red S on both surfaces after 3 weeks of induction. The positively stained area in the bright field images was analyzed using ImageJ software.

Lineage-specific Responsiveness Analysis. Raw reads were aligned and mapped to the human hg38 ENSEMBL genome using bowtie2. Transcripts per million (TPM) for each condition were estimated from mapped reads using RSEM.⁶³ The lineage-specific responsiveness coefficient, R_{LS} was defined as the average transcriptional response to an osteogenic differentiation stimulus of cells on the pillar surface compared to the flat surface. Genes with similar initial prestimulated expression, based on their quantile of $\log_2(E_{\text{Induced}}/E_{\text{control}})$, are grouped and their change in average expression in response to the stimulus is quantified in flat and pillar populations for initially under-expressed and overexpressed genes. Further, GO analysis was done on DE genes with $p\text{-value} < 0.05$ and $|FC| > 1.5$ in induced cells compared to control cells to evaluate the transcriptional malleability in biological processes that are involved in early differentiation.

Statistical Analysis. Some of the results are shown as mean $\pm$ S.D. using a bar plot. The others were shown using the box and whisker plots that represent median values (horizontal bars), 25th to 75th percentiles (box edges), and minimum to maximum values (whiskers), with all points plotted. Statistical analysis was performed using Kyplot software (version 2.0 beta 15). We also reported standard error of the mean (SEM) with mean values obtained from imaging experiments. Statistical significance among each group was determined by unpaired t-test (two-sided) and a value of $p < 0.05$ was considered to indicate a statistically significant difference. For RNA-Seq analysis, the cutoff for determining significantly differentially expressed genes was a p -value less than 0.05. All experiments presented in the manuscript were repeated at least as two independent experiments with replicates.

Reporting Summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Declarations

Data availability

The main data supporting the results in this study are available within the main manuscript and its Supplementary Information. The raw and analyzed datasets generated during the study are available from the corresponding authors upon reasonable request.

Code availability

Custom codes used in this study can be accessed on GitHub following this link (<https://github.com/BME2021/LineageSpecificResponsiveness/blob/main/LineageSpecificResponsiveness.ipynb>)

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Contributions

X.W., V.A., V.B., and G.A. designed the experiments. X.W. and V.A. performed the experiment and analyzed the data. Y.L. helped with the EM data collection and analysis. R.V. helped with GSEA and transcriptional response analysis, P.P. did the RNA-seq differential gene expression analysis, and J.F. assisted with the RNA-seq analysis. S.J. helped with transcriptional data interpretation and analysis. E.R. and R.B. helped with ChromTEM sample preparation. N.R. and C.D. helped with cell culture and sample preparation. X.W., V.A., S.J., B.J., P.N., V.B., and G.A. wrote the manuscript. All the authors discussed the results and reviewed the manuscript.

Competing Interests

The authors declare no competing interests.

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Figures

Figure 1

Micropillars alter the nuclear architecture and mechanics of hMSCs. A. Phase-contrast image of square micropillars with pillar size and space of 5 x 5 μm . B. SEM images showing the side and section view of the micropillars. C. Typical DAPI staining images of cell nuclei on flat and pillar substrates. D. Nuclear shape index of cells on flat (n=273 cells) and pillar substrates (n=295 cells). N=4 experiments. E. F-actin and vinculin staining images of cells on flat and pillar substrates. F. Representative orthogonal view of cell nuclei on flat and micropillar surfaces showing their 3D structures. G. and H. are volume and surface area of cell nuclei on flat (n=33 cells) and micropillar (n=34 cells) surfaces. N=3 experiments. I. Lamin A/C staining images of cell nuclei on flat and micropillar surface. Solid red arrows indicate strong staining of Lamin A/C; hollow red arrows indicate weak staining of Lamin A/C; yellow arrows indicate wrinkles of Lamin A/C surrounding micropillars. J. and K. show fluorescence intensity plot of Lamin A/C along selected regions (yellow rectangles 1 and 2 in C) in xy and (yellow rectangles in I) xz plane in the control and deformed nucleus. L. and M. show the average Lamin A/C intensity ratio at xy and xz plane of cell nuclei on flat (n=25 cells) and micropillar surface (n=26 cells). N=3 experiments. N. Schematic summary of influence of micropillars on the polarization of nuclear Lamin A/C and mechanical state. On flat surfaces, actin-caps that formed above the nucleus compress it and the cell adhere to the substrate beneath which leads to burial of the epitope in polymerized lamin A/C at basal NE. On micropillars, the pillar structures prevented the expansion of the nucleus and led to its compression, and provided adhesion with cells that induced multimerization of lamin A/C around micropillars and induced horizontal polarization of NE. (**p<0.01). (***)

Figure 2

Nuclear deformation-induced changes in histone modifications. A. Immunostaining images and B. western blot images of H3Ac and H3K27me3 in cell nuclei on flat and micropillar surfaces. Total histone H3 and GAPDH are shown as a control. C. Relative change of H3Ac and H3K27me3 expression compared to total H3 expression in cells. The relative expression level on flat surface was normalized to be 1 (N=3 experiments). D. Immunostaining images of HDAC 3 in cells on flat and micropillar surfaces. White and yellow arrows indicate staining signal in the nucleus and cytosol, respectively. E. Intensity ratio of nuclear HDAC3 to cytoplasmic HDAC3 fluorescence intensity per area of cells on flat (n=232 cells) and micropillar (n=238 cells) surfaces. N=3 experiments. F. Immunostaining images of EZH2 in cells on flat and micropillar surfaces. White and yellow arrows indicate staining signal in the nucleus and cytosol, respectively. G. Relative change of EZH2 expression compared to total H3 expression in cells. The relative expression level on flat surface was normalized to be 1 (N=3 experiments). ($*p<0.05$, $*p<0.05$, $***p<0.001$).

Figure 3

Micropillars cause downregulation of developmental processes in hMSCs. A. Volcano plot displaying differentially expressed genes in hMSCs seeded on micropillars compared to the flat surface. A negative fold change indicates genes downregulated in the micropillar condition compared to flat. B. Top 100 Differentially Expressed genes in cells seeded on the flat surface compared to control based on absolute log fold change in hMSCs. C. Gene Ontology (GO) analysis for the top 20 most enriched processes in cells seeded on micropillars compared to a flat surface in hMSCs. (List of genes annotated to each process can be found in Table S2) A network analysis of the top 20 canonical pathways gene sets D. depleted, and E. enriched in pillars found using GSEA. Processes and pathways in D and E are colored by cluster-ID, where nodes that share the same cluster-ID are typically close to each other (terms with a similarity, Kappa Scores > 0.3 are connected by edges).

Figure 4

Chromatin Packing Scaling Decreases in Deformed hMSC Nuclei. PWS microscopy of hMSCs and osteogenic differentiating hMSCs show a significant change in average chromatin packing scaling, D in deformed nuclei compared to control. Stem cells on A. flat and B. micropillar surfaces show changes in packing domains (red clusters). C. Micropillars deform cell nuclei and significantly decrease average D in stem cells for $n=111$ hMSCs on flat surface and $n=110$ hMSCs on pillar surface. $N=4$ experiments. D. ChromTEM images of 50 nm thick resin sections of hMSCs seeded on flat and micropillar surface. The technique resolves nucleoli, nuclear speckles, and mitochondria at high resolution. Orange arrows show the location of micropillars. E. Spatial autocorrelation function (ACF) of chromatin density in the log-log scale for the whole nucleus, and peripheral chromatin. We

obtained the chromatin packing scaling by performing a linear regression of the ACF in the log-log scale within the ranges of 80-200 nm for the whole nucleus and 50-75 nm for peripheral chromatin. F. Chromatin packing scaling shows the significant difference for whole-cell nuclei and periphery of nuclei cultured on flat (n=20 hMSCs) and pillar surfaces (n=12 hMSCs), indicating a drastic change in the chromatin organization. (**p<0.01, ****p<0.0001). N=2 experiments.

Figure 5

Micropillars Increase Lineage Specific Response by decreasing D in hMSCs. A. Mesenchymal stem cells seeded on a flat surface (high D) and micropillar surface (low D) (SF and SP, respectively) were induced to differentiate towards osteogenic lineage (IF and IP, respectively). E denotes expression rate in the respective conditions used to evaluate lineage-specific responsiveness coefficient, RLS, a measure of response to differentiation induction (osteogenic differentiation in this case) on the pillar surface compared to flat surface. B. Lineage-specific transcriptional response due to differentiation induction increases in low D cells for 3323 differentially expressed genes with $p\text{-adjusted} < 0.05$ and C. 1513 genes with $p < 0.05$ and $|\text{fold change}| > 1.5$ for high (orange) and low initial expressions (purple) in the control cells (SF). Here, each dot represents 126 genes (in B) and 57 genes (in C). CPMC Model predictions for initially lowly expressed genes and initially highly expressed genes are shown in orange and purple respectively. D. A network of top 20 pathways and processes identified from all DE genes ($p\text{-adj} < 0.05$ and $|\text{FC}| > 1.5$) in induced cells compared to stem cells on a flat surface: colored by cluster-ID, where nodes that share the same cluster-ID are typically close to each other (terms with a similarity, Kappa Scores > 0.3 are connected by edges). E. Lineage-specific transcriptional response due to differentiation induction increases in low D cells for all DE genes with $p\text{-adj} < 0.05$ and $|\text{FC}| > 1.5$ (where each blue dot represents 150 genes) and genes in development-specific processes (highlighted cluster in green) where each green dot represents 57 genes, and bone development genes (highlighted in red) where each dot represents 12 genes. (List of genes annotated to each process can be

found in Table S4) F. Log2 Fold Change in gene expression of DKK1 (Wnt inhibitor), DVL3, and DVL1 (Wnt conductor), as well as TLE4 (Wnt co-repressor) on flat and micropillar substrates upon osteogenic induction. A negative fold change indicates downregulation in micropillars compared to the flat surface. G. Representative immunofluorescent images of β -catenin and RUNX2 in cells. H. and I. show the intensity ratio of nuclear/cytoplasmic β -catenin and RUNX2 in cells. $n=187$ and 199 cells on flat and micropillar surfaces, respectively. $N=3$ experiments. J. ALP staining images and ALP activity analysis of cells after 7-day osteogenic differentiation induction ($N=4$ experiments). K. Relative gene expression of ALP and IBSP tested after osteogenic induction for 7 days of cells on flat and micropillar substrates ($N=3$ experiments). (* $p < 0.05$, *** $p < 0.001$).

Figure 6

Schematic illustration of the effects of micropillars on osteogenic differentiation of hMSCs. Compared to that on a flat surface, cell nuclei on micropillars had an anisotropic distribution of lamin A/C. A decrease in chromatin packing scaling in the whole nucleus was observed using PWS and ChromTEM. Epigenetic changes like an increase in repressive histone modifications like H3K27me3 and a decrease in activating modifications H3 acetylation were observed on micropillars. Such epigenomic changes may lead to downregulation of the majority of genes on micropillars. As a consequence of the decrease in chromatin packing scaling, lineage-specific responsiveness upon osteogenic induction was increased in micropillars.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [TableS1MicropillarvsFlatStemMSCsDifferentialExpressionSignificant.xlsx](#)
- [TableS2GeneOntologyMetascapePillarvsFlatDowregulatedGenesStemXInducedConditions.xlsx](#)
- [TableS3Top20OntologyGeneSetsFlatXMicropillars.csv](#)
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